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PRINCIPAL INVESTIGATOR: Wen Y. Chen, Ph.D.

CONTRACTING ORGANIZATION: Clemson University
Clemson, South Carolina 29634-5702

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Wen Y. Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Clemson University
Clemson, South Carolina 29634-5702

E-Mail: wenc@clemson.edu

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13. ABSTRACT ***(Maximum 200 Words)***

There were three tasks proposed for the second year of this project. Majority of the tasks have been accomplished. We were able to produce and purify large amount of hPRl and hPRL-G129R using *E. coli* expression system. The purified proteins were used to carry out *in vitro* as well as *in vivo* characterization of the hPRL antagonist (four manuscripts and six abstracts were published). We further confirmed that the antagonistic effects of hPRL-G129R in breast cancer cells are probably through the inhibition of STATs phosphorylation, caspase activation, or TGFs modulation. Based upon our initial studies, we have found that that proposed multi-cell receptor level comparison using conventional binding assay was not sensitive enough for differentiate the difference. We carried out real time RT-PCR studies to quantify the PRL-receptor level (manuscript published). One negative result is also included in this report. We were unable to produce hPRL-BP in *E.coli* expression system. We are trying to use a new strain of *E. coli* from Novogen to overcome this problem in the coming year.

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Introduction

Human breast cancer is the most predominant malignancy with the highest mortality rate in women from western society. Many risk factors have been identified for this disease. Several lines of evidence strongly linked human prolactin (hPRL) to breast carcinogenesis. In this proposal, two novel approaches have been designed to generate hPRL receptor specific antagonists. First approach is to adopt a site-directed mutagenesis strategy by which hGH receptor antagonist, hGH-G120R, was discovered, to produce a mutated hPRL, hPRL-G129R, and use it as hPRL receptor **blocker**. The other approach is to design and produce a soluble form of extra-cellular domain of hPRL receptor namely hPRL binding protein (hPRL-BP), and use it to **sequester** autocrine/paracrine effects of hPRL. After cloning of hPRL and hPRL-BP cDNAs, mutation will be made in hPRL cDNA to generate hPRL-G129R. Human PRL, hPRL-G129R and hPRL-BP cDNAs will be produced and purified using *E. coli* protein expression system. The purified proteins will then be used to test its bioactivities in multiple human breast cancer cell lines and two non-breast origin human cancer cell lines (as controls) for receptor binding, inhibition of phosphorylation of the STATs protein induced by hPRL (as an indicator for intracellular signaling), and inhibition of human breast cancer cell proliferation. We hope that these two novel approaches will ultimately result in generation of hPRL antagonists that could be used to improve human breast cancer therapy.

Body

There are three original tasks proposed for the second year of this project.

---- Production, purification and quantification of hPRL, hPRL-G129R and hPRL-BP.
---- Competitive receptor binding assays, STATs assays
---- Initiating cell proliferation assays using hPRL, hPRL-G129R and hPRL-BP on twelve human cancer cells.

We have produced and purified at least 900mg each of hPRL and hPRL-G129R during this period. The proteins have been used to further explore the mechanisms of apoptosis induced by hPRL-G129R in human breast cancer cells. We have published four peer reviewed manuscripts and six abstracts (national meeting presentations) during this period. We have found that hPRL-G129R is able to inhibit hPRL induced STAT phosphorylation (Cataldo et al, 2000). We have also found that hPRl-G129R modulates TGFs (up-regulation of TGF-b and down regulation of TGF-a), and Caspases (Ramamoothy et al., 2000). By using a protocol we developed in the lab, we have also found that hPRL up-regulates bcl-2, an apoptosis inhibitor (Beck and Chen, manuscript in press).

The fourth paper is related to the levels of hPRL receptor in multiple breast cancer cells (Peirce and Chen, 2001). When we were carrying out hPRL receptor binding assays, we found out that the method we originally proposed was not sensitive enough to differentiate the difference among those cell lines express low levels of hPRL receptor. To solve this problem, we have used a real time PCR technology to quantified the hPRL receptor levels in multiple breast cancer cell lines we have cultured (see attachment for detail).

A main negative result in this period is that we were still unable to produce hPRL-BP in *E. coli* system despite many trials using different *E. coli* stains. We believe it probably has something to do with the codon usage. In this regard, we have recently found a new *E. coli*. strain called "Rosetta TM provided by Novegen. The Rosetta host strains are BL21 derivatives designed to enhance the expression of eukarytic proteins that contain codons rarely used in E. coli. Thus the Rosetta strains provide for so-called "universal" translation. We hope this approach will give us some break through in terms of hPRL-BP production.

Key Research Accomplishment for the Second Year.

We have further confirmed the antagonistic effects of hPRL-G129R through *in vitro* studies. In our recent publications, we have demonstrated that the inhibitory effects of hPRL-G129R is possibly through the inhibition of STAT phosporylation (Cataldo et al., 2000), TGF modulation (Ramamoothy et al., 2000), and caspase induction (Ramamoothy et al., 2000). We have also explored the molecular mechanism of hPRL-G129R induced apoptosis. We found that it is possible that PRL serves as an apoptosis inhibitor through the induction of Bcl-2. (Beck and Chen, in press). We have also published a manuscript regarding the PRL receptor levels in multiple cell lines (Peirce and Chen, 2001).

Reportable Outcomes

Four manuscripts, six abstracts/meeting presentations (see appendix A-E):

A. Cataldo L, Chen, NY. Li, W, Wagner, TE, Sticca RP and Chen,WY Inhibition of the Oncogene STAT3 by a Human Prolactin (PRL) Antagonist is a PRL Receptor Specific Event. Int. J. Oncology 17:1179-1185, 2000.

B. Ramamoorthy P, Sticca RP, Wagner TE, Chen WY In vitro Studies of a Prolactin Antagonist, hPRL-G129R, in human breast Cancer Cells. Int. J. Oncology 18:25-32, 2000.

C. Peirce S and Chen WY Quantification of Prolactin Receptor mRNA in Multiple Human Tissues and Cancer Cell Lines by Real Time RT-PCR. J. Endocrinology. J Endocrinol Oct;171(1):R1-4, 2001

D. Beck MT, Holle H, Chen WY Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling. Biotechniques 30 (10) in press.

E. Six abstracts in recent meeting presentations

1. *In Vivo* Studies of the Anti-tumor Effects of a Human Prolactin Antagonist, hPRL-G129R in Nude Mice Chen, N.Y., Li, W., Cataldo, L., Sticca, R.P., Wagner, T.E. and Chen, W.Y. AACR 2001
2. A novel design of targeted endocrine and cytokine therapy for human breast cancer G.R. Zhang, W. Li[2], L. Holle, N.Y. Chen and W.Y. Chen. Endo 2001. (oral presentation).

3. Characterization of a human prolactin antagonist/ granulocyte macrophage colony stimulating factor fusion protein. L. Holle, W. Li, N.Y. Chen and W.Y. Chen. Endo 2001

4. Profiling of apoptosis related genes responding to prolactin and its antagonist in human breast cancer cells. M.T. Beck, L. Holle and W.Y. Chen. Endo 2001

5. Real time RT-PCR analysis of relative prolactin receptor (PRLr) levels in human cancer cell lines. S.K. Peirce, R.B. Westberry and W.Y. Chen. Endo 2001

6. Enhancement of the inhibitory effects of suppressor of cytokine signaling 3 (SOCS3) protein in cancer cells by VP22 Q. Yuan, S.K. Peirce, R.B. Westberry and W.Y. Chen. Endo 2001.

One Ph. D. student (Dr. Helen Zhang) and two master students (Mr. Ryan Westberry and Ms. Qiu Yuan) were graduated during this year (partially supported through this award).

Conclusions:

In our second year of work, we have further confirmed that that hPRL-G129R acted as a hPRL antagonist in human breast cancer cell lines. We have also made considerable progress elucidating the mechanisms involved in hPRL-G129R induced apoptosis. We will complete the study regarding hPRL-G129R as planned during the remaining of the project. In the mean time, we will continue the effort to produce hPRL-BP.

Appendix A

Inhibition of the Oncogene STAT3 by a Human Prolactin (PRL) Antagonist is a PRL Receptor Specific Event.

Cataldo L, Chen, NY. Li, W, Wagner, TE, Sticca RP and Chen,WY

Int. J. Oncology 17:1179-1185, 2000

Inhibition of oncogene STAT3 phosphorylation by a prolactin antagonist, hPRL-G129R, in T-47D human breast cancer cells

L. CATALDO[1], N.Y. CHEN[1], Q. YUAN[2], W. LI[2], P. RAMAMOORTHY[2], T.E. WAGNER[1,2], R.P. STICCA[1] and W.Y. CHEN[1,2]

[1]Oncology Research Institute, Greenville Hospital System, Greenville, SC; [2]Department of Microbiology and Molecular Medicine, Clemson University, Clemson, SC, USA

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Abstract. We have previously demonstrated that a hPRL antagonist (hPRL-G129R) was able to inhibit PRL induced breast cancer cell proliferation through induction of apoptosis. In the present study, we test the hypothesis that the inhibitory effect of hPRL-G129R in breast cancer cells occurs, at least in part, through the inhibition of oncogene STAT3 activation. We first demonstrated that STAT5 and STAT3 could be activated by either hGH or hPRL in T-47D breast cancer cells. Although the patterns of STAT5 activation by hGH and hPRL are similar, we observed a nearly 10-fold greater efficacy of hPRL in STAT3 activation as compared to that of hGH. More importantly, we have demonstrated that activation of STAT3 by hPRL could be inhibited by hPRL-G129R. Since T-47D cells coexpress GHR and PRLR, an attempt was made to dissect the molecular events mediated through hGHR or hPRLR using mouse L-cells expressing a single population of receptors (hGHR or hPRLR). To our surprise, only STAT5, not STAT3 phosphorylation was observed in these L-cells. In conclusion, our results suggest that: a) STAT3 is preferably activated through hPRLR in T-47D cells; b) hPRL-G129R is effective in inhibiting STAT3 phosphorylation; and c) the mechanism of STAT3 activation is different from that of STAT5.

Correspondence to: Dr W.Y. Chen, Oncology Research Institute, Greenville Hospital System, 700 W. Faris Road, Greenville, SC 29605, USA
E-mail: wchen@ghs.org

Abbreviations: hPRL, human prolactin; hGH, human growth hormone; hPRL-G129R, human prolactin antagonist; PRLR, prolactin receptor; GHR, growth hormone receptor; STAT, signal transducer and activator of transcription; Cys, cysteine; L-GHR, mouse L-cells expressing GHR; L-PRLR, mouse L-cells expressing PRLR; E2, estradiol; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; bGH, bovine GH; FBS, fetal bovine serum; CSS, charcoal stripped serum; IPTG, isopropyl-thiogalactoside; IRMA, immunoradiometric assay; MET, methionine

Key words: prolactin antagonist, breast cancer, STAT3

Introduction

STAT (signal transducers and activators of transcription) proteins are important transcriptional regulators in the cell, and have been studied in great detail (1-6). Seven STAT genes have been identified that encode eight different STATs; STAT1α, 1ß, 2, 3, 4, 5a, 5b and 6 (1,2,4,7). Each STAT plays an important, yet different role in signal transduction. STAT proteins have two main functions that include signal transduction in the cytoplasm and activation of transcription in the nucleus (2,4). STATs are usually activated in response to a ligand/receptor interaction. Binding of cytokines or hormones to their respective receptors stimulates the Janus kinase family of proteins which then phosphorylate STAT proteins on a specific tyrosine residue at the COOH terminus (4). Homo- or heterodimers are formed between the phosphorylated tyrosine of one STAT molecule and the SH2 domain of another STAT molecule. These dimers translocate into the nucleus, by a mechanism that is unknown, and function as transcription factors by binding to their recognition sequences and regulating the target gene expression (3-5).

STAT5 and STAT3 have been shown to be critical in mammary gland development by homologous recombination gene disruption studies in mice (8,9). Given the importance of STATs in the control of mammary gland developmental processes and their intimate association with cytokines and hormones, it is not surprising that inappropriate activation of STATs has been found in human breast cancer and other malignancies (10-12). The autocrine/paracrine effects of certain ligands including PRL or GH have been reported to increase activity of tyrosine kinases and therefore the hyperactivity of STATs (13-16). STAT3, which was initially identified in interleukin 6 induced signaling pathways (17,18), recently was shown to be significant in cancer. This is based on the finding that certain forms of cancer and tumor cell lines show constitutively active STAT3 (19-24) and that STAT3 can transform cells (25-29). In addition, a naturally occurring mutant form of STAT3, termed STAT3ß, was found to be able to suppress the growth of B16 melanoma cells *in vitro* and *in vivo* (30). STAT3ß has a mutation in the carboxy region of STAT3, and therefore it is not able to activate transcription. More recently, several lines of evidence clearly elucidate the functional role of STAT3 as an oncogene (31). A constitutively

active form of STAT3 was developed in which amino acid residues important for STAT3 dimerization were replaced with Cys. This Cys substitution resulted in a constitutively dimerized (via homodimerization through disulfide bonds) and therefore active form of STAT3. The mutated form of STAT3 had the ability to transform cells and to induce tumor formation *in vivo*. It was also shown that constitutively active STAT3 leads to increased c-*myc* and cyclin mRNA which is important for cell proliferation (32-34) and increased Bcl-X_L mRNA, which is an anti-apoptotic factor (35,36). The above lines of evidence point to the possibility of using STAT3 as a therapeutic target.

There is a high incidence of breast cancer in women from Western countries, but the cause of breast is still unknown. Recently, the relationship between hPRL and breast cancer has been re-emphasized (37-41). After the finding of locally produced PRL by the mammary gland and the up-regulation of PRLR in breast cancer samples (37,40,41), hPRL is now considered as an autocrine/paracrine growth factor that contributes to breast cancer development. It is believed that local production of PRL by breast cancer cells results in autocrine/paracrine stimulation of PRL receptors that perhaps leads to uncontrolled cell proliferation (14,16). STATs 3 and 5 are involved in PRL mediated signal transduction (7), therefore suggesting a role for these factors in breast cancer.

In our recent studies, we have demonstrated that a single amino acid substitution at position 129 of hPRL (hPRL-G129R) resulted in a true hPRL receptor antagonist in human breast cancer cell based assays (42). We have shown that: a) hPRL and E2 exhibit additive stimulatory effects on human breast cancer cell proliferation, suggesting that these two stimuli act together through different mechanisms to promote cell proliferation; b) hPRL-G129R binds to the hPRLR with an affinity similar to that of wild-type hPRL; c) hPRL-G129R inhibited breast cancer cell proliferation; and d) when anti-estrogen (4-OH-tamoxifen) and anti-PRL (hPRL-G129R) agents were applied simultaneously, there was an additive inhibitory effect (42). We further investigated the mechanism of the inhibitory effects of hPRL-G129R. Using multiple human breast cancer cell lines, we also demonstrated that hPRL-G129R was able to induce apoptosis in a dose dependent manner as determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The main goal of our current study is to further elucidate the role of hPRL in breast cancer, in particular the relationship between hPRL, STAT5 and STAT3. In view of the fact that STAT3 has been shown to be an oncogene, we are especially interested to see if hPRL-G129R is able to inhibit the activation of STAT3 in human breast cancer cells.

Materials and methods

Cell lines used for STAT3 and STAT5 phosphorylation studies. T-47D cells, a human breast cancer cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). They were maintained in phenol red-free RPMI 1640 media supplemented with 10% fetal bovine serum (Life Technologies Inc.). L-GHR cells and L-PRLR cells were established by extracting mRNA from T-47D breast cancer cells using the Micro-Fast Track 2.0 kit available from Invitrogen Corp. (Carlsbad, CA). The full-length cDNA encoding hPRLR and hGHR were cloned using RT-PCR (using a RT-PCR kit from Promega Corp.). Full-length hPRLR and hGHR cDNAs were then cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequences were confirmed using an ABI 310 Genetic Analyzer. Both cDNA sequences were identical to that published in GeneBank (data not shown). The hGHR and hPRLR cDNAs were then sub-cloned into a pIG-Met expression vector containing the mouse metallothionein regulatory sequences and bGH polyA signal. This expression vector has been used in many of our previous studies (43-45).

TK (thymidine kinase) and APRT (adenine phosphoribosyl transferase) mouse L-cells were used to establish stably transfected hGHR and hPRLR L-cells as described previously (44,45). Briefly, L-cells were transfected with the plasmids using lipofectin (Life Technologies). HAT (hypoxathine aminopterin thymine) resistant colonies were isolated and propagated in tissue culture flasks. Positive L-hGHR and L-hPRLR cells were identified by RT-PCR and subsequently verified by receptor binding assay (data not shown). The stable cell lines with high expression levels were then propagated and maintained in DMEM supplemented with 10% FBS (Life Technologies Inc.). All cell lines were grown at 37°C in an atmosphere containing 5% CO_2.

E. coli production and purification of hPRL-G129R. hGH and hPRL used in this study were a kind gift from Dr A.F. Parlow (National Hormone and Pituitary Program, NIH). The hPRL-G129R used in this study was produced from *E. coli* according to published protocols (46,47) with modifications. Briefly, BL21(DE3) cells (Novagen, Madison, WI) were transformed with hPRL-G129R plasmid using the calcium chloride method. The transformant was spread on an ampicillin plate, and grown overnight at 37°C. The LB seed culture was inoculated with 6-10 colonies and grown overnight. The following day an LB growth culture was generated by inoculation of 5% of the seed culture and grown for ~2.5 h at 37°C with agitation. IPTG (Fisher Scientific) was then added to the culture (1 mM final concentration) to induce expression of hPRL-G129R and incubated for an additional 4 h. Bacteria were pelleted and resuspended in a solution containing 0.2 M $NaPO_4$ pH 8.0, 10 mM EDTA, and 0.5% Triton X-100. The resuspended bacteria were lysed with a 550 Sonic Dismembrator (Fisher Scientific). The hPRL-G129R product, which is in the form of an inclusion body, was pelleted at 12,000 g for 15 min and resuspended in 0.2 M $NaPO_4$ pH 7.0, 1% v/v ß-mercaptoethanol, 8 M urea for refolding. The refolding process consisted of dialyzing the protein against decreasing amounts of urea and ß-mercaptoethanol in the presence of 50 mM NH_4HCO_3 pH 8.0 for three consecutive days. The sample was first filtered through a 0.22 micron filter (VWR), degassed and then purified by a Q-Sepharose anionic exchange column (Pharmacia, Piscataway, NJ) using a FPLC system (Pharmacia, Piscataway, NJ). The concentration of hPRL-G129R purified from FPLC was determined using the Prolactin IRMA kit (DPC, Los Angeles, CA). The purity of hPRL-G129R was over 90% on SDS-PAGE using the silver staining method (Biorad, Hercules, CA). The hPRL-G129R produced by this

method has an extra Met at the N-terminus as compared to wild-type PRL.

Extraction of protein from cultured cells for STAT3 and STAT5 assays. Twenty-four hours prior to protein extraction T-47D cells were resuspended in RPMI media containing 10% charcoal stripped serum (CSS; Hyclone, Logan, UT), plated into 6-well plates and grown to confluency. L-cells expressing either GHR or PRLR were resuspended in DMEM containing 10% FBS and plated into 6-well plates to confluency. On the day of treatment, T-47D cells were depleted for 30 min in RPMI containing 0.5% CSS and L-cells expressing either GHR or PRLR were depleted for 2 h in DMEM. Cells were treated for 20 min with the appropriate amount of hGH (NIH), hPRL (NIH, National Hormone and Pituitary Program) or hPRL-G129R (produced in our laboratory). Cells were then washed with ice cold PBS (Life Technologies) and 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM PMSF; 1 µg/ml aprotinin and 1 µg/ml leupeptin; and 1 mM Na_3VO_4) was added to each well. Cells were incubated on an orbital rotator for 15 min and then lysate was transferred to a 1.5 ml Eppendorf tube. Lysate was gently passed through a 21 gauge needle 5-6X to shear genomic DNA and then placed on ice 20 min. Lysate was spun at 14,000 rpm for 20 min at 4°C in a microcentrifuge.

Preparation of cell lysates for STAT3 and STAT5 analysis. Thirty-five µl of cell lysate (65-70 µg) was added to 15 µl of 3X SDS-PAGE sample buffer. Total protein obtained from cultured cells was approximately equal for all cell lines used as determined by the Bradford protein assay (Biorad, Hercules, CA). Samples were heated for 5 min at 100°C, and then analyzed on a 4-15% gradient gel (Biorad, Hercules, CA). Protein was transferred to Hybond nitrocellulose membrane (Amersham, Arlington Heights, IL) for 2.5 h at 12 W. A high molecular weight rainbow marker (Amersham, Arlington Heights, IL) was used to determine the protein size as well as the success of the transfer.

Analysis of STAT3 and STAT5 protein levels. The protocol used to determine STAT3 levels or STAT5 levels was obtained from Upstate Biotechnology Institute (UBI, Lake Placid, NY). The protocol was altered slightly, and is described below. After protein transfer, membranes were washed briefly with distilled water and then blocked for 20 min in PBS containing 3% non-fat powdered milk (Biorad, Hercules, CA) for STAT3 analysis and in TBS containing 5% non-fat powdered milk, and 0.05% Tween-20 for STAT5 analysis. Membranes were then incubated in either STAT3 antiserum (UBI, Lake Placid, NY) at a concentration of 2 µg/ml or a 1:4,000 dilution of STAT5a antiserum and a 1:4,000 dilution of STAT5b antiserum (UBI, Lake Placid, NY) overnight at 4°C with constant agitation. Membranes were washed twice with distilled water (5 min/wash), and were incubated in a 1:2,000 dilution of goat anti-rabbit horseradish peroxidase secondary antibody (Biorad, Hercules, CA) for 2 h at room temperature with constant agitation. After secondary antibody incubation, membranes were washed once with distilled water, once with PBS containing 0.05% Tween-20, and once with distilled water. Membranes were developed for 1 min using enhanced chemiluminescence reagents (ECL; Amersham, Arlington Heights, IL). Membranes were then exposed to Kodak MR film (Fisher).

STAT3 and STAT5 tyrosine phosphorylation analysis. The same overall protocol was followed as for the analysis of STAT3 and STAT5 total protein levels. Membranes were incubated overnight with constant agitation in mouse phospho-STAT3 antiserum (UBI, Lake Placid, NY) at a concentration of 1.7 µg/ml or in mouse phospho-STAT5a/b antiserum (UBI, Lake Placid, NY) at a concentration of 1.5 µg/ml. Phospho-STAT3 antiserum was specific for phosphorylated tyrosine 704, and phospho-STAT5 antiserum was specific for phosphorylated tyrosine 694 for STAT5a and 699 for STAT5b. After primary antibody incubation, membranes were incubated in a 1:2,000 dilution of goat anti-mouse horseradish peroxidase conjugated secondary antibody (Biorad, Hercules, CA) for 2 h at room temperature. Membranes were washed as described above and developed for 1 min using ECL reagents. Membranes were exposed to Kodak MR film.

Results

Dose response studies for STAT5 and STAT3 phosphorylation in T-47D breast cancer cells. Dose response studies for STAT3 and STAT5 phosphorylation were first carried out in T-47D cells, which coexpress hGHR and hPRLR. T-47D cells were treated with increasing concentrations of either hPRL or hGH. It is clear that STAT5 can be maximally activated by either hPRL or hGH in T-47D cells at a dose of approximately 250 ng/ml (Fig. 1). Activation of STAT3 was also observed in T-47D cells treated with either hPRL or hGH (Fig. 2). However, the levels of STAT3 phosphorylation induced by hPRL at a concentration of 50-100 ng/ml were compatible to the levels of STAT3 phosphorylation induced by hGH at 500-1,000 ng/ml (Fig. 2).

hPRL-G129R inhibits STAT3 phosphorylation induced by hPRL in T-47D cells. hPRL-G129R inhibits hPRL induced STAT3 phosphorylation in T-47D cells. Fig. 3 shows the results of competition studies in which T-47D cells were treated with hPRL, hPRL-G129R, or a combination of the two in different concentrations. It is clear that hPRL-G129R is not active in terms of STAT phosphorylation (either STAT5 or STAT3; Fig. 3). At a 1:1 ratio of hPRL-G129R:hPRL, phosphorylation of STAT3 was significantly inhibited, whereas at a 5:1 ratio, phosphorylation of STAT3 is completely inhibited (Fig. 3b). hPRL-G129R appears to inhibit STAT3 phosphorylation to a greater extent than STAT5 phosphorylation (Fig. 3a).

STAT5, but not STAT3, is activated in L-GHR or L-PRLR cells. Because T-47D cells coexpress PRLR and GHR, we wanted to look at STAT3 and STAT5 phosphorylation in the presence of a single population of receptors using L-GHR or L-PRLR cells. STAT5 phosphorylation was detected at very high levels in both L-GHR or L-PRLR cells [there is no activation in parental L-cells as described previously (48) suggesting the activation is through transfected human

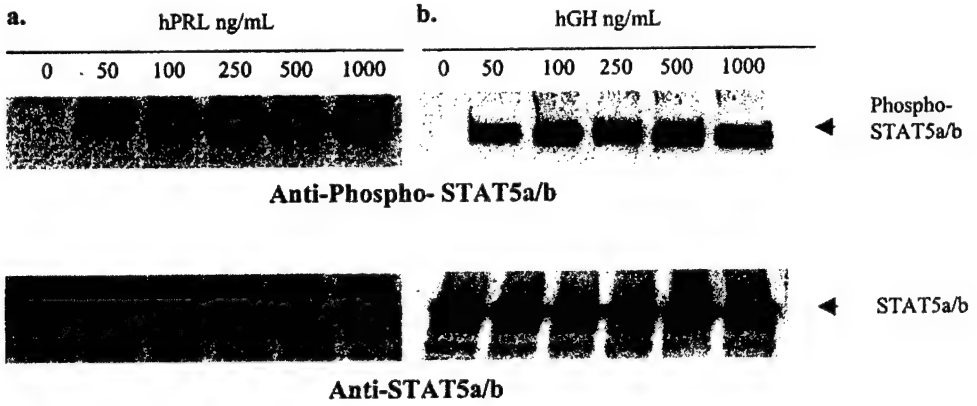

Figure 1. Dose response studies for STAT5 phosphorylation in T-47D cells. Cells were treated with increasing amounts of either hPRL or hGH. Total cellular protein was extracted from cells and subject for gradient SDS-PAGE followed by Western analysis with phospho-STAT5 antiserum (a and b, top) and anti-STAT5a and STAT5b antiserum (a and b, bottom).

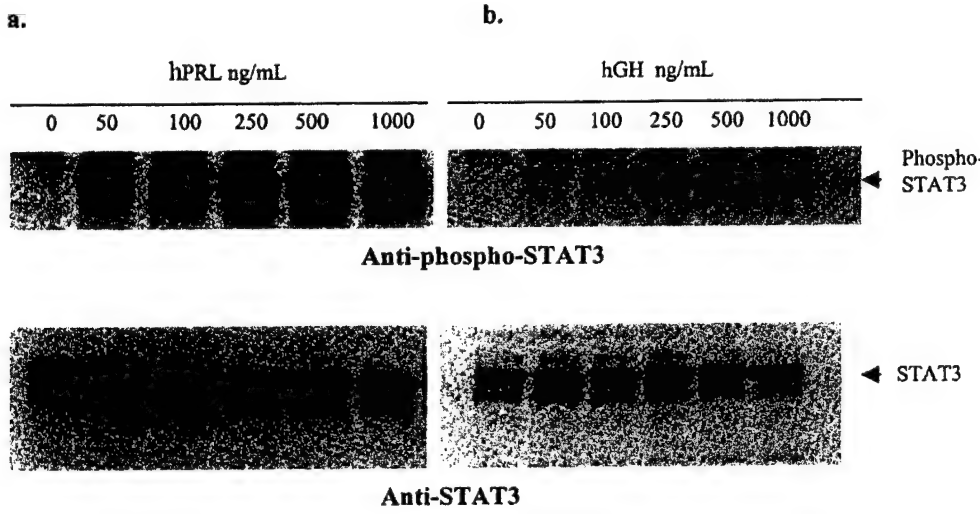

Figure 2. Dose response studies for STAT3 phosphorylation in T-47D cells. Cells were treated with increasing amounts of either hPRL or hGH. Total cellular protein was extracted from cells and subject for gradient SDS-PAGE followed by Western analysis with phospho-STAT3 antiserum (a and b, top) and anti-STAT3 antiserum (a and b, bottom).

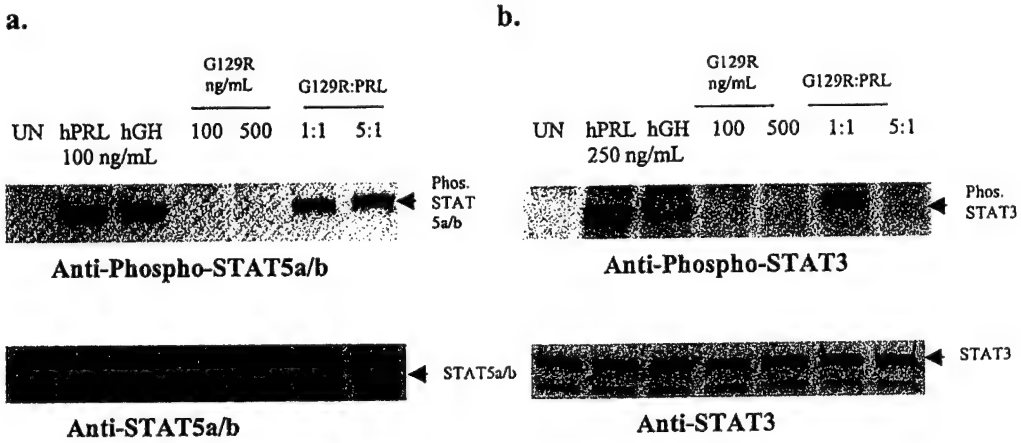

Figure 3. Inhibition of hPRL induced STATs phosphorylation by hPRL-G129R in T-47D cells. Cells were treated for 20 min with hPRL, hGH, or hPRL + hPRL-G129R. The amount of hPRL used for STAT5 competition studies was 100 ng/ml whereas 250 ng/ml was used for STAT3 studies since at these concentrations the phosphorylation of STATs reached maximal level. The untreated (UN) cells were used as a control. A 1:1 ratio or a 5:1 ratio of hPRL-G129R:hPRL was used for treatment. Total cellular protein was then extracted from cells and analyzed by Western blotting with either phospho-STAT5a/b antiserum (a, top) or phospho-STAT3 antiserum (b, top) or STAT5 antiserum (a, bottom) or STAT3 antiserum (b, bottom).

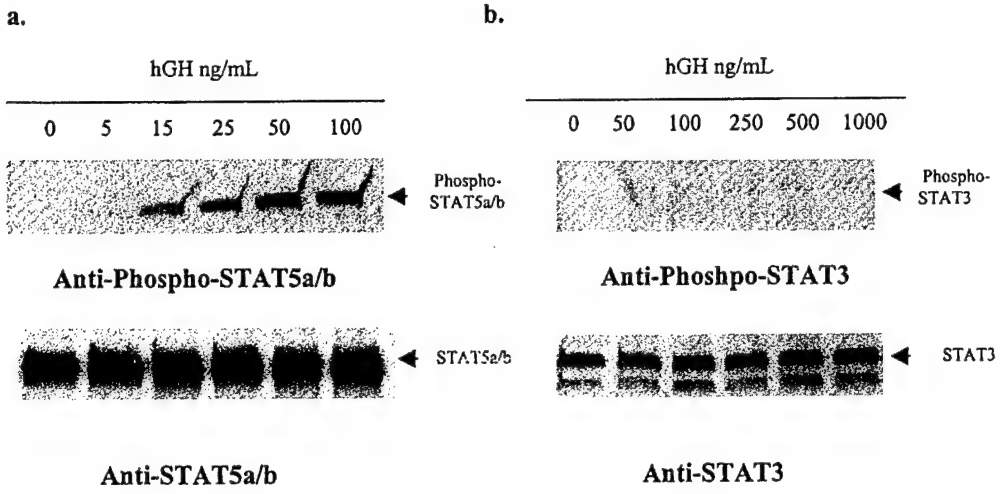

Figure 4. Dose response studies for STAT5 and STAT3 phosphorylation in L-GHR cells. Cells were treated with increasing amounts of hGH and then total protein was extracted. Maximum STAT5 phosphorylation is detected in L-GHR cells at a concentration of 50 ng/ml of hGH (a, top). STAT3 phosphorylation was not detected in L-GHR cells treated with hGH (b, top). Protein levels were equal in each case as indicated by Western analysis with either STAT3 antiserum or STAT5a/b antiserum (a and b, bottom).

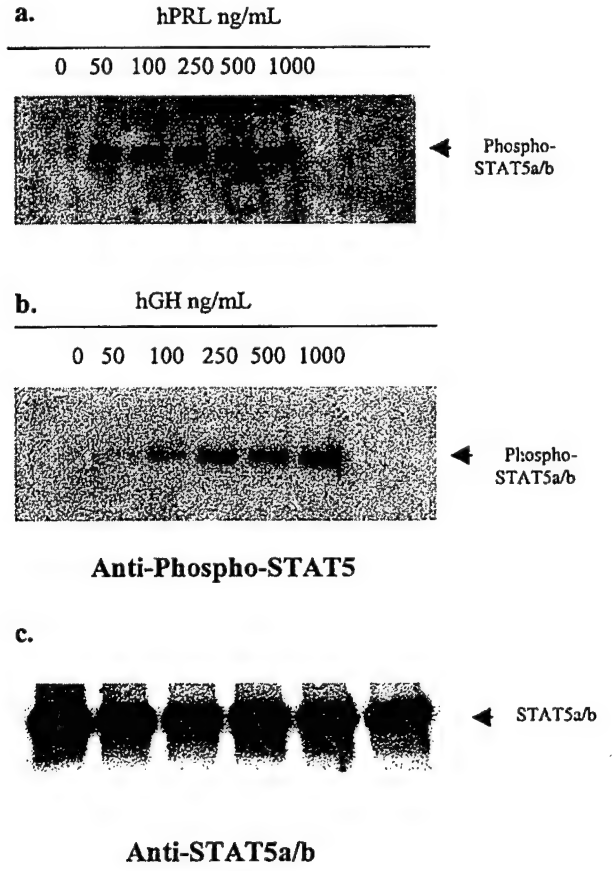

Figure 5. Dose response studies for STAT5 phosphorylation in L-PRLR cells. L-PRLR cells were treated with increasing amounts of hPRL and hGH. The cellular protein was then extracted. Protein was analyzed by Western blotting with either phospho-STAT5 antiserum (a and b) or antiserum against STAT5 protein (c). Maximum STAT5 phosphorylation is seen at a hPRL concentration of 250 ng/ml (a, top). Maximum STAT5 phosphorylation is seen at a hGH concentration of 1,000 ng/ml (b, middle). Panel c showing that an equal amount of protein was loaded in each well. This panel is representative of cells treated with either hPRL or hGH.

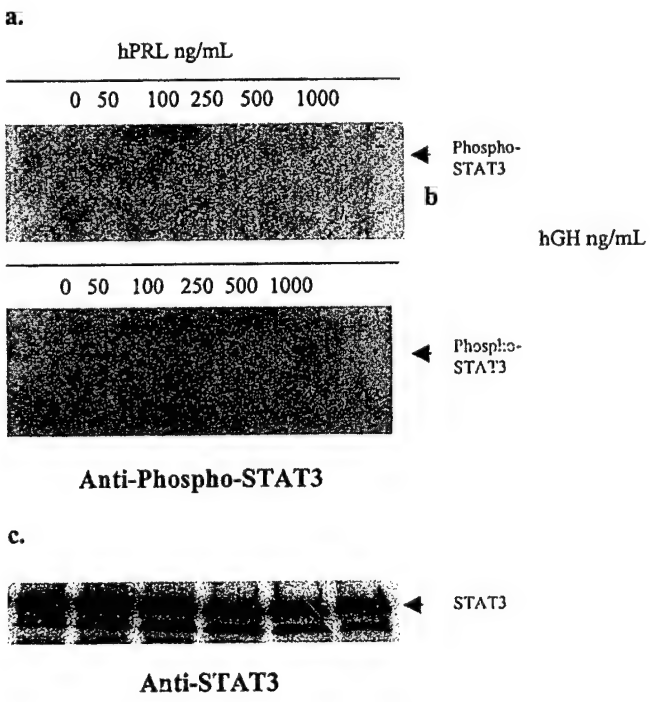

Figure 6. Dose response studies for STAT3 phosphorylation in L-PRLR cells. L-PRLR cells were treated with increasing amounts of hPRL and hGH. The cellular protein was extracted. Protein was analyzed by Western blotting with either phospho-STAT3 antiserum (a and b) or antiserum against STAT3 protein (c). STAT3 phosphorylation is not detected in L-PRLR cells either treated with hPRL (a, top) or hGH (b, middle). Equal amounts of STAT3 protein were seen at a relatively high level in each lane (c, bottom). Panel c is representative of cells treated with either hPRL or hGH.

receptors; Figs. 4a, 5a and b]. 50 ng/ml of hGH is able to induce maximum phosphorylation of STAT5 in L-GHR cells (Fig. 4a). On the other hand, STAT5 phosphorylation was observed in L-PRLR cells when these cells were stimulated by either hPRL or hGH at the compatible concentration range (Fig. 5a and b). Interestingly, the concentration required for hGH to induce maximum STAT5 phosphorylation in L-PRLR cells is much higher as compared to that needed in L-GHR cells (Figs. 4a and 5b). As expected,

STAT5 phosphorylation was not observed in L-GHR cells treated with hPRL, since hPRL does not bind to hGHR (data not shown).

To our surprise, when L-GHR or L-PRLR cells were treated with hGH or hPRL, STAT3 phosphorylation was not detected (Figs. 4b, 6a and b) despite the fact that relatively high levels of STAT3 protein are present (Figs. 4b and 6c).

Discussion

The role of hPRL in human breast cancer has recently been re-emphasized (37-41). In our previous studies, we demonstrated that a hPRL antagonist with a single amino acid substitution, hPRL-G129R, was able to inhibit hPRL induced human breast cancer cell proliferation through induction of apoptosis (42). In this study, we tested the hypothesis that the inhibitory effects of hPRL-G129R on human breast cancer cells are mediated, at least in part, through the inhibition of STAT phosphorylation, and in particular, STAT3 phosphorylation. The results from T-47D breast cancer cells demonstrated that both STAT5 and STAT3 are tyrosine phosphorylated in response to either PRL or GH (Figs. 1 and 2). A similar pattern of STAT5 activation induced by hGH or hPRL was observed (Fig. 1). However, hPRL is much more efficient in activating oncogene STAT3 as compared to that of hGH in T-47D cells (Fig. 2). There is approximately a 10-fold difference between these two ligands (the levels of STAT3 phosphorylation induced by 50-100 ng/ml of hPRL are equivalent to that induced by 500-1,000 ng/ml of hGH, Fig. 2). Although lacking direct evidence, we speculate that the activation of STAT3 by hGH is probably due to the fact that hGH is able to bind to hPRLR. The difference in efficacy between hGH and hPRL in inducing STAT3 phosphorylation probably reflects the difference between a homologous system (hPRL/hPRLR interaction) and a heterologous system (hGH/hPRLR interaction).

We further demonstrated that hPRL-G129R is able to competitively inhibit STAT3 phosphorylation induced by hPRL in T-47D cells (Fig. 3). At a 5:1 ratio (hPRL-G129R: hPRL), hPRL-G129R can completely inhibit STAT3 phosphorylation. It is of interest that inhibition of STAT3 phosphorylation by hPRL-G129R is much more efficient as compared to its ability to inhibit STAT5 phosphorylation (Fig. 3). This data further strengthens our speculation that STAT3 activation is more specific to the hPRLR pathway.

It is known that T-47D cells coexpress GHR and PRLR. In an attempt to differentiate the STAT phosphorylation events mediated through hGHR or hPRLR, we cloned full length hGHR and hPRLR cDNA from T-47D cells and established mouse L-cells with a single population of either hGHR or hPRLR. As expected, STAT5 activation was observed in both cell lines when exposed to respective ligands (Figs. 4a and 5a). As expected hGH is able to activate STAT5 in L-hPRLR cells but hPRL was inactive in L-hGHR cells. It is worthy to point out that the concentration needed for hGH to elicit strong STAT5 phosphorylation in L-PRLR cells is much higher than that in L-GHR cells. The data provide further evidence that a heterologous ligand/receptor interaction is less efficient than a homologous ligand/receptor system. To our surprise, however, we did not observe any STAT3 phosphorylation event in either of the cell lines even at the highest ligand concentration (Figs. 4b, 6a and b) despite the fact that relatively high amounts of STAT3 protein are present in these L-cells (Figs. 4b and 6c). One explanation for this obvious difference between the activation of STAT5 and STAT3 in these stable L-cell lines is that the crucial factors that link the events between ligand/receptor activation and STAT phosphorylation are unique for individual STAT activation. The factors that link ligand/receptor activation to STAT5 phosphorylation are common or can be shared between human cells (T-47D) and mouse L-cells. However, the factors that link ligand/receptor activation to STAT3 phosphorylation are either species specific or are missing or mutated in L-cells. It is also necessary to determine if the activation of STATs seen in T-47D breast cancer cells, but not in fibroblast mouse L-cells, is a breast cancer cell specific phenomenon. If this is true, the status of STAT3 phosphorylation might provide a clinical indication of the application of the hPRL antagonist.

In conclusion, cancer is a disease in which one of the hallmarks is uncontrolled cell proliferation and aberrant signal transduction (49,50). For example, cancer cells may overexpress a specific signal transduction factor; such is the case in the constitutive activation of oncogene STAT3 (31). It is possible that the constant presence of hPRL (an autocrine/paracrine growth factor) in the local breast tumor microenvironment makes the breast cancer cells rely heavily on factors involved in PRL signaling pathways, such as STAT3. The data presented in this study demonstrates that hPRL-G129R is able to specifically inhibit STAT3 phosphorylation in breast cancer cells. Although further clinical studies are needed to demonstrate the relevance of STAT3 activation and breast cancer, we believe that hPRL-G129R could potentially be a valuable addition to breast cancer therapy based on its abilities to inhibit PRL induced breast cancer cell proliferation, induce apoptosis and inhibit oncogene STAT3 phosphorylation.

Acknowledgments

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Appendix B

In vitro Studies of a Prolactin Antagonist, hPRL-G129R, in human breast Cancer Cells.

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In vitro studies of a prolactin antagonist, hPRL-G129R in human breast cancer cells

PREVEEN RAMAMOORTHY[2], ROBERT STICCA[1], THOMAS E. WAGNER[1,2] and WEN Y. CHEN[1,2]

[1]Oncology Research Institute, Cancer Center, Greenville Hospital System, Greenville, SC 29605;
[2]Department of Microbiology and Molecular Medicine, Clemson University, Clemson, SC 29681, USA

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Abstract. Human prolactin (hPRL) has been shown to be one of the important survival/growth factors that promotes the proliferation of breast cancer cells in an autocrine/paracrine manner. In our recent studies, we demonstrated that a hPRL antagonist with a single amino acid substitution mutation (hPRL-G129R) was able to inhibit breast cancer cell proliferation via induction of apoptosis (1). In this study three independent yet related experiments were carried out regarding the effects of hPRL-G129R in breast cancer cells. We investigated the possible mechanism(s) of hPRL-G129R induced apoptosis in breast cancer cells. It is well documented that transforming growth factors (TGF) in conjunction with hormones such as estrogen and PRL play a major role in modulating the proliferation and apoptosis of mammary cells. We first investigated the relationships between hPRL/hPRL-G129R and TGFs. We show that hPRL is able to down-regulate TGFß1 (apoptotic factor) secretion and up-regulate TGFα (survival factor) secretion in a dose-dependent manner in T-47D cells. More importantly the hPRL antagonist up-regulates TGFß1 and down-regulates TGFα secretion. When hPRL-G129R was applied together with hPRL, it blocked the effects of hPRL. Secondly, we tested the possible involvement of caspases in hPRL-G129R induced apoptosis. We have shown that caspase-3 is activated by hPRL-G129R at a concentration of 250 ng/ml in T-47D breast cancer cells. Thirdly, we explored the additive effects of an anti-neoplastic drug, cisplatin, with the hPRL-G129R in T47D breast cancer cells. We show that cisplatin and hPRL-G129R when applied together resulted in about 40% growth inhibition in T-47D cells.

Correspondence to: Dr Wen Y. Chen, Oncology Research Institute, Greenville Hospital System, 700 W. Faris Road, Greenville, SC 29605, USA
E-mail: wchen@ghs.org

Abbreviations: hPRL, human prolactin; CDDP, cis-diammine-dichloroplatinum; FBS, fetal bovine serum; CSS, charcoal stripped serum; ATCC, American Type Culture Collection; TGFα and TGFß, transforming growth factor α and ß; ELISA, enzyme linked immunosorbent assay

Key words: prolactin antagonist, breast cancer, apoptosis, caspase-3, transforming growth factors, cisplatin

Introduction

Human prolactin (hPRL) has been shown to be one of the important survival/growth factors that can mediate the proliferation of breast cancer cells in an autocrine/paracrine manner. hPRL has been linked to breast cancer by several lines of evidence: a) biologically active PRL has been found in breast cancer cells (2); b) hPRL receptor expression levels are up-regulated in breast cancer cells/tissues (3); c) PRL transgenic mice have a high breast cancer rate (4); and d) a hPRL antagonist inhibits the proliferation of breast cancer cells by induction of apoptosis as demonstrated in our previous studies (1). These finding join the growing body of evidence that PRL is indeed one of the major players in the genesis/progression of breast cancer. In this study we investigate the possible mechanism(s) of hPRL-G129R induced apoptosis in breast cancer cells.

Apoptosis is a genetically regulated process of cell death and is an integral part of the development and homeostasis of all organisms. The mammary gland apoptosis occurs in sequential waves during development and involution beginning with each pregnancy and ending with each weaning. The regulation of normal breast development is dependent on hormones such as estrogen (E2) and PRL. In addition, growth factors such as TGFß and α are also implicated in the development of the breast. After weaning, withdrawal of PRL (along with other factors) results in one of the dramatic examples of apoptosis: remodeling of the breast that accompanies post-lactational involution. This highly regulated balance between proliferation, differentiation, and regression (apoptosis) requires fine control by hormones and growth factors, as well as cross-talk between epithelial cells and stromal fibroblasts of the mammary gland (5). In transgenic mouse studies, overexpression of TGFα blocks the mammary gland remodeling process, suggesting that TGFα may be acting as a survival factor for the mammary epithelium (6). In contrast, transgenic mice that overexpress TGFß showed increased mammary epithelium apoptosis throughout mammary development, suggesting that TGFß may be acting as an apoptotic factor for the mammary epithelium (6). Hormones such as PRL and E2 have also

been reported to modulate and cross-talk with the TGFs. For instance, TGFα has been shown to activate the mouse mammary tumor virus long terminal repeat in a similar fashion as PRL (7). E2 stimulates the secretion of TGFα and reduces the levels of TGFß1 in breast cancer cells (8,9) and PRL has been shown to inhibit the activity of TGFß in a murine hybridoma model (10). Interestingly, it has also been reported that TGFß1 inhibits PRL synthesis in the lactotroph cells through an autocrine/paracrine mechanism (11).

On the other hand, tamoxifen (TAM), an estrogen receptor (ER) antagonist, up-regulates TGFß1. This induction of TGFß1 is believed to play an important role in TAM induced apoptosis in breast cancer cells (12). In addition, plasma levels of TGFß are increased in women treated with TAM, an effect that appears correlated with its anti-tumor effects (5). TGFα is down-regulated by pure ER antagonists such as ICI 182,780 (13). Taken together, in mammary epithelial cells, TGFß acts as an apoptotic factor as it can be up-regulated by anti-cancer drugs and TGFα acts as a survival factor as it can be up-regulated by hormones that promote breast cancer cell proliferation such as E2 and PRL. Therefore any anti-breast cancer drug that can differentially modulate TGFs, specifically by up-regulation of TGFß (an apoptotic factor) and down-regulation of TGFα (a survival factor) could be very valuable in breast cancer therapy.

Cells undergoing apoptosis exhibit shrunken pyknotic nuclei as well as other characteristic changes such as blebbing. Molecular analyses of apoptotic cells can demonstrate characteristic DNA fragmentation, activation of specific 'death inducing' cellular genes and specific cellular proteases called caspases (14). These changes almost invariably involve chromatin condensation and its margination at the nuclear periphery, extensive double-stranded DNA fragmentation, and cellular shrinkage and blebbing. There is evidence that caspases contribute to the drastic morphological changes of apoptosis by proteolysing and disabling a number of key substrates, including the structural proteins gelsolin, PAK2, focal adhesion kinase, and rabaptin-5. Caspase-3 is one of the key caspases involved in DNA fragmentation (15). Caspase-3 initiates apoptotic DNA fragmentation by proteolytically inactivating DFF45 (DNA fragmentation factor-45)/ICAD (inhibitor of caspase-activated DNase), which releases active DFF40/CAD (caspase-activated DNase), the inhibitor's associated endonuclease. Thus, caspase-3 is the primary inactivator of DFF45/ICAD and therefore the primary activator of apoptotic DNA fragmentation (16). In view of the pivotal role played by caspase-3 in DNA fragmentation we wanted to determine if caspase-3 activation plays a part in hPRL-G129R induced apoptotic DNA fragmentation in breast cancer cells.

In our previous study (1) we demonstrated that the efficacy of growth inhibition of breast cancer cells was almost doubled when tamoxifen (an anti-estrogen agent) was combined with hPRL-G129R (an anti-prolactin). In this study we explored the *in vitro* effects of combining cisplatin, an anti-neoplasic chemotherapeutic drug along with hPRL-G129R as a potential combination therapeutic strategy. Cisplatin is a platinum-containing broad activity anti-neoplastic and alkylating agent effective against malignancies of the testes, ovaries, bladder, oesophagus, head and neck and lung (17). Recently, cisplatin has been reported to have a number of important therapeutic characteristics and has been used in combination therapy regimens. For example, cisplatin has been shown to immunosensitize tumor cells to Fas mediated apoptosis (18). Another study concluded that combination therapy with cisplatin and herceptin, a humanized monoclonal body directed against HER2, results in significant antitumor activity with the potential for reducing toxicity in metastatic breast cancer patients (19). Cisplatin has also been shown to improve the efficacy of gene therapy in malignancies of the head and neck, ovary, prostate and breast (20).

Materials and methods

Cell culture. The T-47D and MCF-7 cell lines obtained from ATCC are positive for both ER and PRL receptors. T-47D cells were grown in RPMI 1640 (phenol red-free to avoid its potential estrogen-like activities) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and ATCC recommended supplements. MCF-7 cells were grown in DMEM (phenol red-free) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and ATCC recommended supplements. Both cell lines were grown at 37°C in a humidity controled atmosphere in the presence of 5% CO_2.

Co-culture experiment. The cell proliferation assay was designed to take advantage of stable mouse L cell lines established by us that produce hPRL-G129R. Increasing numbers of L cells (or L-hPRL-G129R cells) in a range of 4,500-27,000 cells/well were co-cultured with a fixed number of MCF-7 cells (9,000/well) in 96-well plates. Simultaneously, a corresponding set of L cells (or L-hPRL-G129R cells) was cultured in a fixed volume of 200 µl in the same plate (without co-culture with MCF-7 cells) as background control. We have previously (1) used this co-culture set-up with T-47D cells. The total volume of the co-culture was 200 µl. The concentrations of hPRL-G129R at the end of 72 h co-culture were measured at 20-200 ng/ml - a concentration that is within the physiological range. Following 24-h, 48-h, or 72-h incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit, Promega Corp. Madison, WI) was added to each well and plates were read at 490 nm using a Bio-Rad benchmark microplate reader. Seventy-two hours incubation time was optimal. The OD of MCF-7 cells was calculated as total OD (OD of MCF-7 plus L, or L-hPRL-G129R cells, respectively) minus the background ODs (L, or L-hPRL-G129R cells alone).

Caspase-3 assay. The breast cancer cells were switched from 10% FBS to 10% charcoal stripped serum (CSS) containing growth media 6 days before the assay. Approximately 2 million breast cancer cells were plated in 10% CSS containing medium growth media. The next day treatments were performed in 1% CSS containing growth media using hPRL-G129R prepared in the lab as described previously (1). A caspase-3 assay kit (ApoAlert CPP32/caspase-3 assay kit-Clontech Corp.) was used to assess the caspase-3 activity colorimetrically using the cell lysates. The specificity of the reaction was verified using a caspase-3 inhibitor (DEVD-fmk).

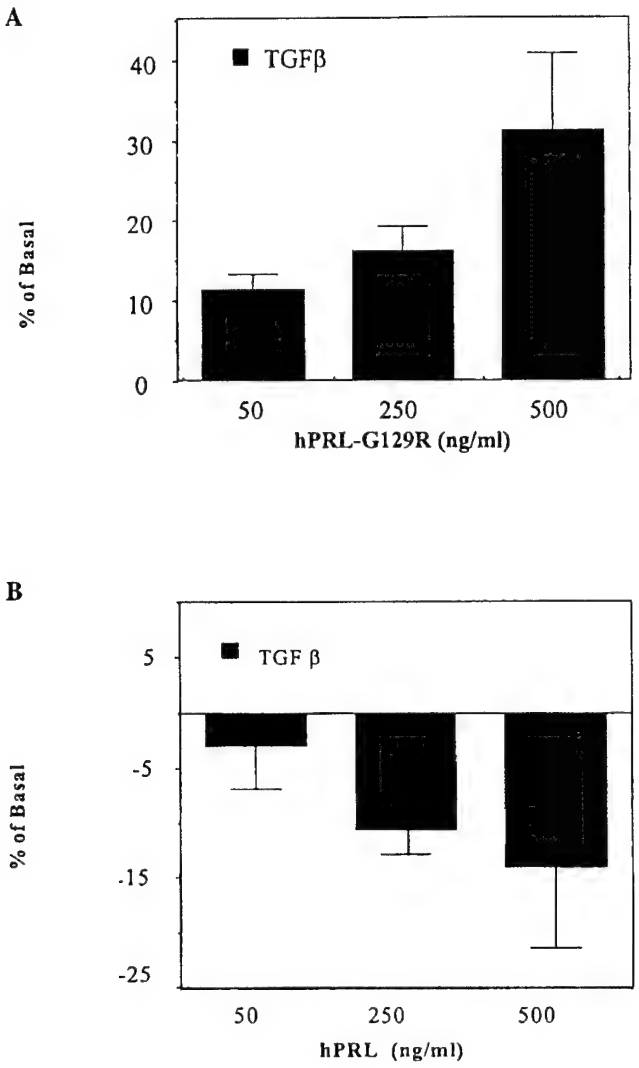


Figure 1. Modulation of TGFß1 by hPRL-G129R (A) and hPRL (B) in T-47D breast cancer cells. Cells were treated with 50 ng, 250 ng and 500 ng/ml of hPRL-G129R or hPRL for 72 h. The induction of TGFß1 by hPRL-G129R is expressed as percent of control above the basal level (A) and the inhibition of TGFß1 is expressed as percent of control below the basal level (B). Each data point represents the mean of at least three experiments. Bars, SD.

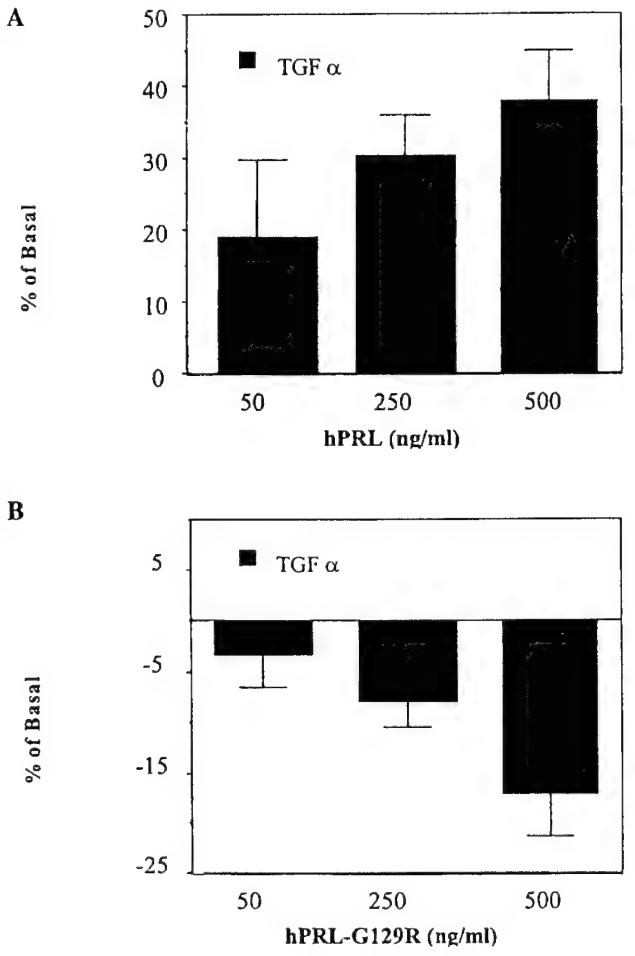


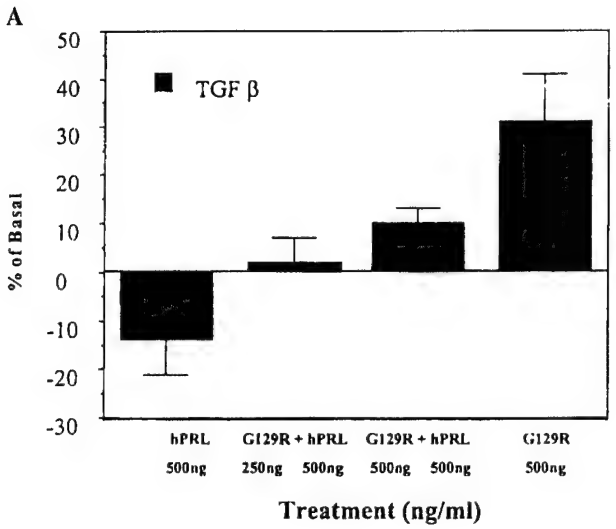
Figure 2. Modulation of TGFα by hPRL (A) and hPRL-G129R (B) in T-47D breast cancer cells. Cells were treated with 50 ng, 250 ng and 500 ng/ml of hPRL for 72 h. The induction of TGFα by hPRL is expressed as percent of control above the basal level (A) and the inhibition of TGFα by hPRL-G129R (B) is expressed as percent of control below the basal level. Each data point represents the mean of at least three experiments. Bars, SD.

ELISA-TGFβ1 and TGFα. Cells were plated in 12-well plates (Corning Costar) using 10% CSS containing growth medium. The following day the cells were starved using serum-free growth medium. Treatments were performed under serum free conditions using hPRL (kindly provided by Dr Parlow, National Hormone & Pituitary Program, NIH) and hPRL-G129R prepared in the lab as described previously (1). The supernatants were collected after a 72-h treatment and were stored as per the manufacturer's instructions. The ELISA's for both TGFß1 and TGFα were carried out using the supernatants from the same experiments. The TGFß1 kit was obtained from Promega Corp. (Madison, WI) and the TGFα kit was obtained from Oncogene Research Products (San Diego, CA).

Cell proliferation assay. The breast cancer cells were switched from 10% FBS to 10% CSS containing growth medium 6 days before the assay. For an individual cell proliferation experiment, 15,000 cells/well were plated in a 96-well plate cultured in 100 µl RPMI-1640 media containing 1% CSS (Collaborative Research, Bedford, MA). Cells were allowed to attach for 12 h, then an additional 100 µl of media containing varying concentrations of hPRL-G129R and cisplatin were added. The hPRL-G129R was prepared as described previously (1). After incubation, MTS-PMS solution was added to each well as per the manufacturer's instructions at 72 h. Plates were read at 490 nm using a Bio-Rad benchmark microplate reader (Bio-Rad Lab., Hercules, CA). Each experiment was carried out in triplicate and was repeated three to six times.

Results

Modulation of transforming growth factors α and β1 by PRL/hPRL-G129R in breast cancer cells. A dose-dependent increase in TGFß1 production was observed with the addition of hPRL-G129R in T-47D cells (Fig. 1A). At a maximal dose of 500 ng/ml, hPRL-G129R increased TGFß1 production to approximately 40% above the basal level (Fig. 1A). A dose-dependent decrease of TGFß1 was observed with the addition of hPRL (Fig. 1B). On the other hand, a dose-dependent increase in TGFα production was induced by



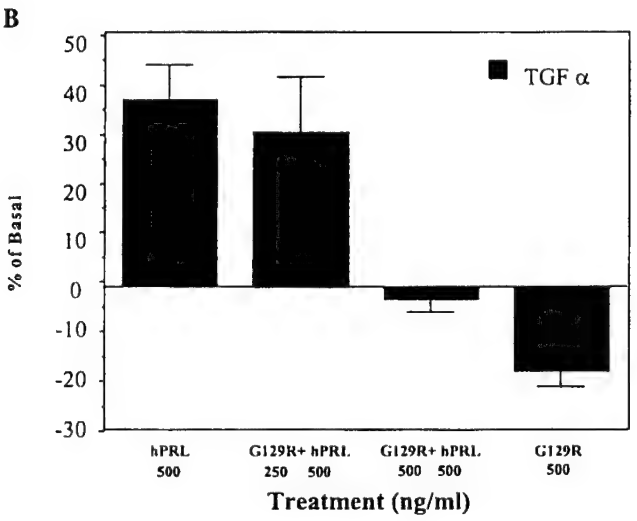


Figure 3. Competitive modulation of TGFß1 and TGFα by hPRL-G129R in T-47D breast cancer cells. Cells were treated for 72 h with a combination of hPRL-G129R and hPRL at a 1:2 and 1:1 ratio. The modulation of TGFß1 (A) or TGFα (B) is expressed as percent of control either above or below the basal level. Each data point represents the mean of at least three experiments. Bars, SD.

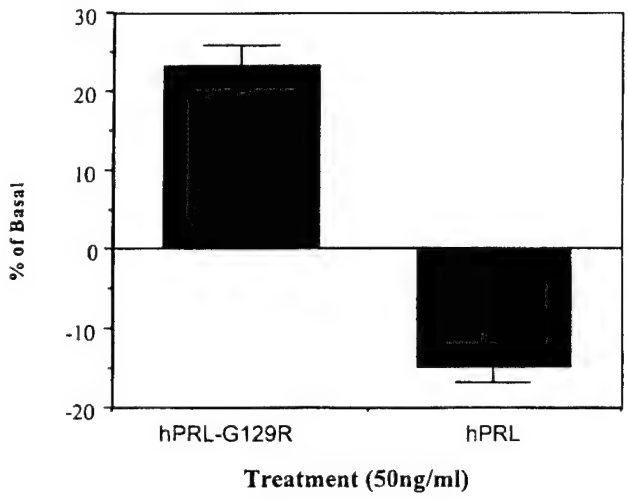


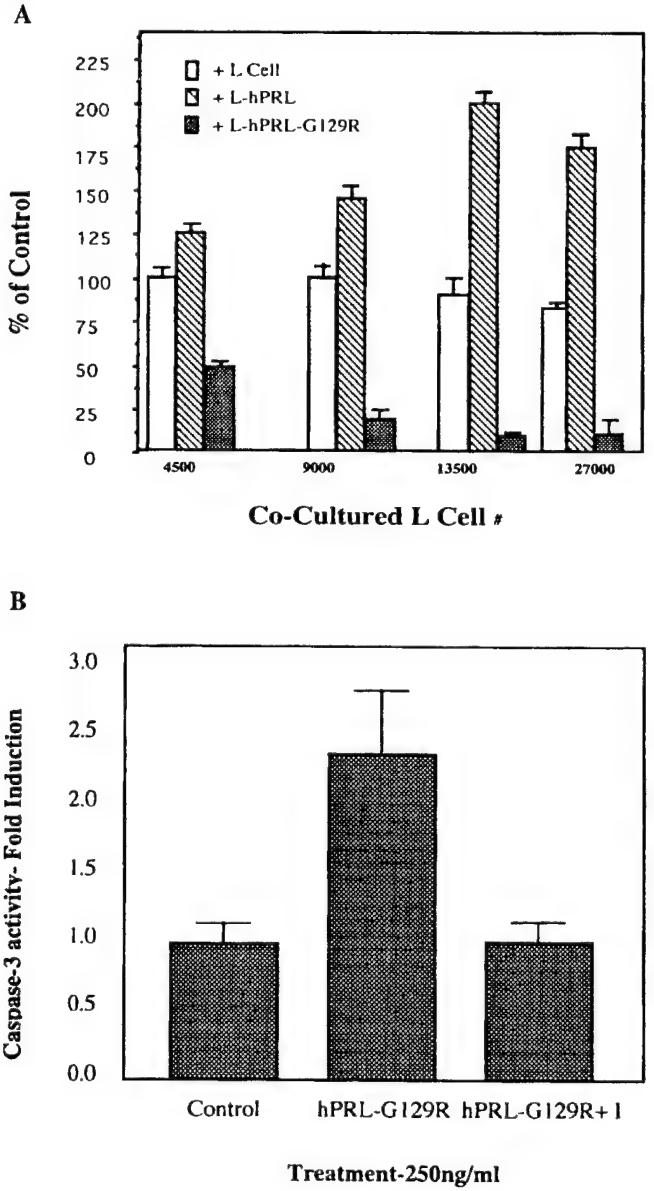
Figure 4. Modulation of TGFß1 by hPRL-G129R and hPRL in MCF-7 breast cancer cells. Cells were treated with 50 ng, of hPRL-G129R or hPRL for 72 h. The induction of TGFß1 by hPRL-G129R is expressed as percent of control above the basal level and the inhibition of TGFß1 is expressed as percent of control below the basal level. Each data point represents the mean of at least three experiments. Bars, SD.

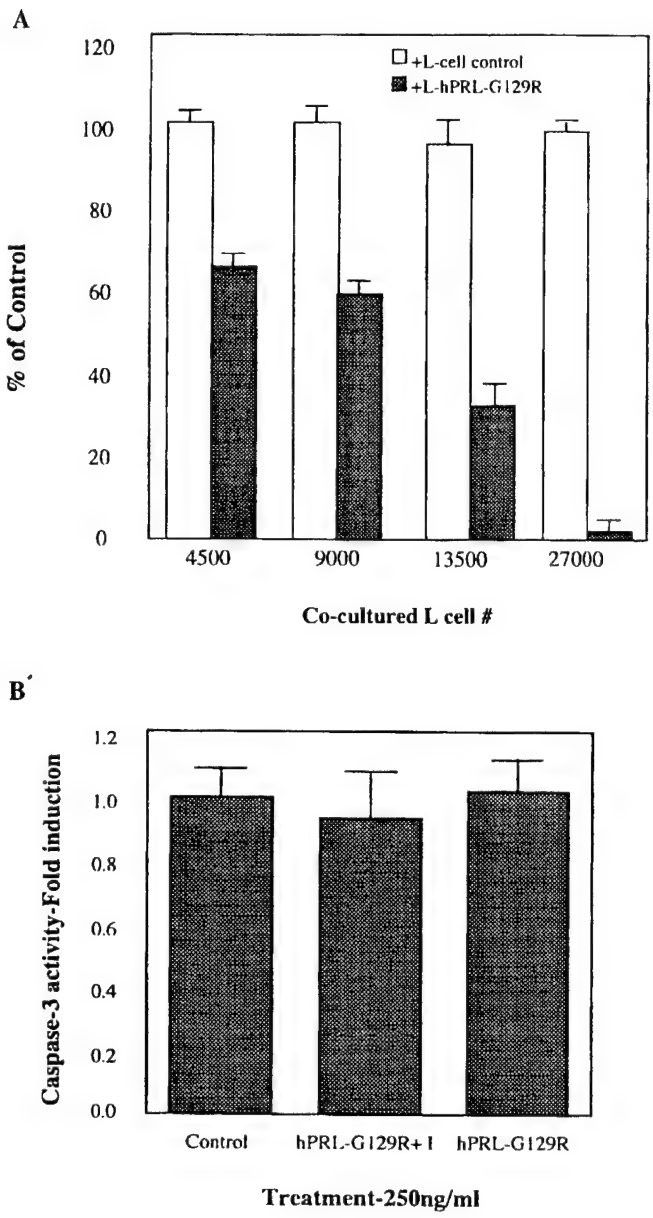
hPRL (Fig. 2A). The maximal increase of TGFα production was approximately 45% above the basal level (Fig. 2A). However, a dose-dependent decrease in TGFα production occurred when T-47D cells were treated with hPRL-G129R (Fig. 2B). The maximal inhibition of TGFα production was approximately 20% below the basal level (Fig. 2B). The TGFß1 level doubled when the concentration of hPRL-G129R was increased from 250 ng/ml to 500 ng/ml in the presence of a fixed amount of 500 ng/ml hPRL (Fig. 3A). In contrast, the TGFα levels were decreased by approximately 40% when the concentration of hPRL-G129R was increased from 250 ng/ml to 500 ng/ml in the presence of a fixed amount of 500 ng/ml hPRL (Fig. 3B). We observed the same trend in TGFß1 modulation in MCF-7 cells (Fig. 4) but surprisingly TGFα was not modulated by hPRL or hPRL-G129R (data not shown) in MCF-7 cells.

hPRL-G129R induced caspase-3 activation in T-47D breast cancer cells. The human prolactin antagonist, hPRL-G129R induces the activation of caspase-3 in T-47D cells at a dose of 250 ng/ml after 2 h treatment (Fig. 5B). Caspase-3 activity is approximately three-fold higher than that of untreated control. The specificity of caspase-3 activation was verified by adding a caspase-3 inhibitor DEVD-CHO along with hPRL-G129R (250 ng/ml). The inhibitor brought the level of caspase-3 activity to the level of control indicating that hPRL-G129R specifically inhibits caspase-3. For the purpose of comparison, we have also shown hPRL-G129R mediated inhibition of cell proliferation (Fig. 5A) and apoptosis (Fig. 5C) from our previous work (1).

Status of caspase-3 activation in MCF-7 breast cancer cells. Previously (1) we showed that hPRL-G129R were induced in both T-47D and MCF-7 cells (Fig. 6C). In view of the fact that caspase-3 was not activated by hPRL-G129R in MCF-7 cells (Fig. 6B), we wanted to determine if inhibition of cell proliferation by hPRL-G129R could also be observed in MCF-7 cells. MCF-7 cells were co-cultured with L cells expressing hPRL-G129R and as a control the MCF-7 cells were co-cultured with untransfected L cells. This co-culture system was used in T-47D cells in our previous work (1). The results (Fig. 6A) show that the L-hPRL-G129R cells are able to inhibit the proliferation of MCF-7 cells in a dose-dependent manner and at the highest dose, a near total inhibition was achieved.

Dose-response inhibitory effects of hPRl-G129R and its additive effects with cisplatin in breast cancer cells. Results from the cell proliferation assay in T-47D cells (Fig. 7) indicate that at a maximal dose of hPRL-G129R there is a 15% inhibition as compared to 25% inhibition with maximal dose of cisplatin. But when both cisplatin and hPRL-G129R





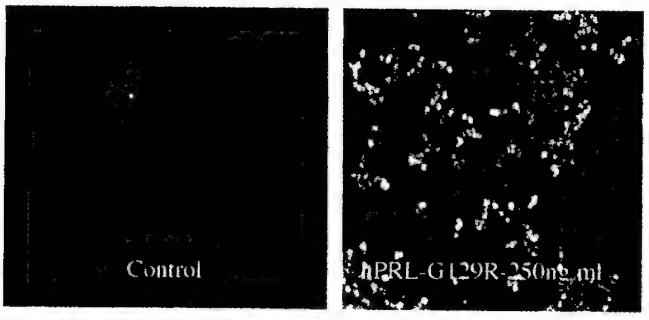


Figure 5. Caspase-3 activation in T-47D breast cancer cells. Induction of Caspase-3 activity by 2 h treatment with 250 ng/ml of hPRL-G129R in T-47D (B). The specificity of induction was verified by using a caspase-3 specific inhibitor DEVD-CHO (represented as I in the graph). For comparison hPRL-G129R mediated cell proliferation inhibition (A) and apoptosis (C) are shown. The data in A and C is from our previous work (1). Each data point represents a mean of at least three independent experiments with triplicates. Bars, SD.

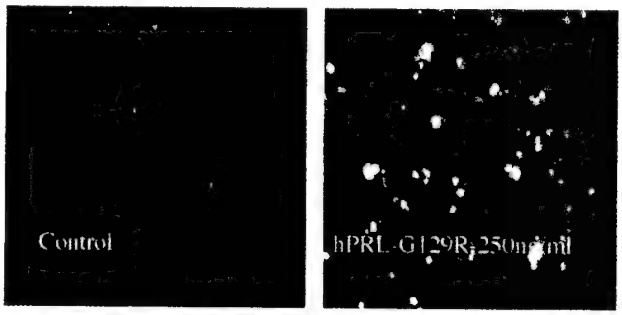


Figure 6. Status of caspase-3 activation in MCF-7 breast cancer cells. Induction of caspase-3 activity by 2 h treatment with 250 ng/ml of hPRL-G129R in MCF-7 (B). The specificity of induction was verified by using a caspase-3 specific inhibitor DEVD-CHO (represented as I in the graph). Fig. 6A shows dose-response inhibitory effects of hPRL-G129R in MCF-7 human breast cancer cells using co-culture method. The x-axis represents the co-cultured L-hPRL-G129R cell numbers. For comparison hPRL-G129R mediated cell proliferation inhibition (A) and apoptosis (C) are shown. The data in C is from our previous work (1). Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

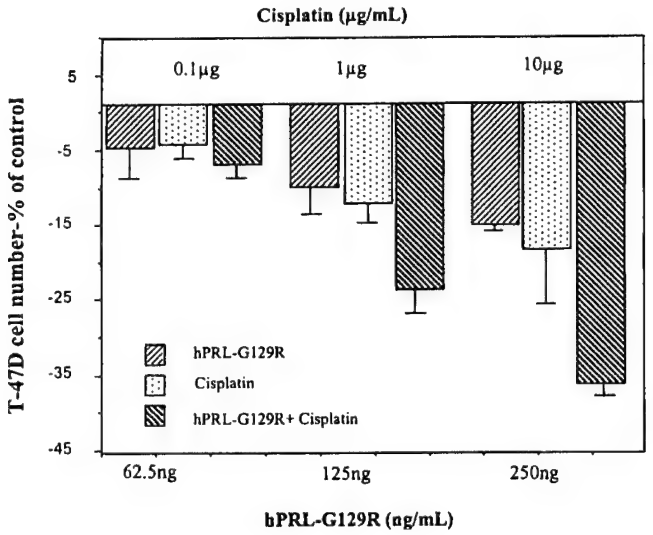


Figure 7. Inhibitory effects of hPRL-G129R and its additive effects with cisplatin in T-47D human breast cancer cell proliferation assay. The x-axis represents the hPRL-G129R concentration either in the absence or presence of cisplatin. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

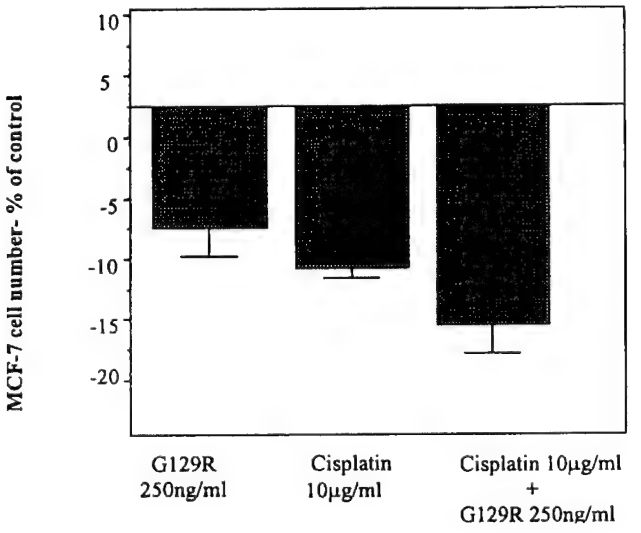


Figure 8. Inhibitory effects of hPRL-G129R and its additive effects with cisplatin in MCF-7 human breast cancer cell proliferation assay. The x-axis represents the hPRL-G129R concentration either in the absence or presence of cisplatin. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

were combined at their respective maximal doses the inhibition of T-47D cells reached about 40%. The same trend was observed in MCF-7 cells (Fig. 8). Thus, the inhibitory effect of cell proliferation by hPRL-G129R and cisplatin appears to be additive.

Discussion

In our previous study we demonstrated that hPRL-G129R, inhibited the proliferation of breast cancer cells through induction of apoptosis (1). In this study we explored the possible mechanisms of hPRL-G129R induced apoptosis in human breast cancer cells, in particular, the roles of TGFs and caspase-3. In addition, we studied the potential additive effects of hPRL-G129R and cisplatin.

In view of the critical role played by TGFs in cell proliferation and apoptosis during mammary gland development (5) and their modulation by anti-estrogens (12,13) we studied the relationship between PRL, hPRL-G129R and TGFs. In this report, we demonstrate that hPRL-G129R up-regulates TGFß1 (an apoptotic factor) and down-regulates TGFα (a survival factor) after a 72 h treatment in T-47D cells (Figs. 1A and 2B) which is opposite to the effects elicited by the treatment of PRL (Figs. 1B and 2A). It is also noteworthy that the pattern of regulation of TGFs by the PRL antagonist, hPRL-G129R, in breast cancer cells is similar to that of anti-estrogens (12,13). Not surprisingly, E2 and PRL were reported to have a similar pattern in modulating TGFs (7-11). In order to assess the competitive nature of hPRL-G129R and hPRL in modulating the TGFs, T-47D cells were treated with increasing amounts of hPRL-G129R in the presence of a constant amount of hPRL. It is evident that hPRL-G129R is able to completely block and partially reverse the effects of hPRL (Fig. 3).

We speculate that the constant presence of PRL in the breast tumor microenvironment is responsible for TGFα up-regulation and TGFα down-regulation and the combination of these two events leads to increased proliferation and decreased apoptosis of breast cancer cells. The addition of hPRL-G129R competitively blocks the effects of PRL, thereby resulting in up-regulation of TGFß and down-regulation of TGFα leading to increased apoptosis and decreased proliferation of breast cancer cells. Although further studies are needed to elucidate the molecular mechanism(s) of hPRL-G129R modulation of TGFs in breast cancer cells, we postulate that there could be cross talk between the signal transduction pathways of PRL and TGFs at the levels of STATs (signal transducers and activators of transcription) and SMADs. STATs and SMADs are two families of transcription factors that are activated in response to respective ligand binding to their membrane bound receptors (21). In hematopoietic cells it has been shown that cytokine signaling through JAK/STAT pathways are generally antagonistic to TGFß, which signals through the SMAD pathway (22). A recent study has shown that IFNγ (a cytokine that signals through JAK/STAT pathway) inhibits the TGFß induced phosphorylation of SMADs in leukemia and fibrosarcoma cells (21). In this study we have shown that PRL, which signals through the JAK/STAT pathway, is able to inhibit TGFß production, suggesting that PRL might be antagonizing TGFß signaling through the inhibition of SMAD phosphorylation. On the other hand TGFα and PRL have both been shown to activate STAT3 in breast cancer cells (Cataldo LA *et al*, and Kelly PA *et al*, p173; Endocrine Society Meeting, 2000). In the present study we show that hPRL up-regulates TGFα production in breast cancer cells, suggesting that the PRL induced STAT3 activation might be involved in TGFα up-regulation.

In our previous study (1) we demonstrated that hPRL-G129R inhibited T-47D cell proliferation (Fig. 5A)

Appendix C.

Quantification of Prolactin Receptor mRNA in Multiple Human Tissues and Cancer Cell Lines by Real Time RT-PCR.

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Quantification of Prolactin Receptor mRNA in Multiple Human Tissues and Cancer Cell Lines by Real Time RT-PCR

SUSAN K. PEIRCE[1], and WEN Y. CHEN[1,2*]

Department of Microbiology and Molecular Medicine, Clemson University, Clemson, SC 29638[1] and Oncology Research Institute, Greenville Hospital System, Greenville, SC 29605[2]

ABSTRACT: Human prolactin (hPRL) has been reported to be involved in breast and prostate cancer development. The hPRL receptor (hPRLR) is expressed in a wide variety of tissues in at least three isoforms. In this study, a one-step real time reverse transcription PCR technique was used to determine relative expression levels of hPRLR mRNA in eleven human breast cancer cell lines, HeLa cells, three prostate cancer cell lines and nine normal human tissues. The housekeeping gene β-actin was used for internal normalization. We demonstrate that hPRLR mRNA is up-regulated in six of the eleven breast cancer cell lines tested when compared to normal breast tissue. Of the cancer cell lines tested, we found that T-47D cells have the highest level of hPRLR mRNA, followed by MDA-MB-134, BT-483, BT-474, MCF-7 and MDA-MB-453 cells. In two breast cancer cell lines (MDA-MB-468 and BT-549), the hPRLR levels were found to be comparable to that of normal breast tissue. Three breast cancer cell lines (MDA-MB-436, MDA-MB-157 and MDA-MB-231) expressed hPRLR mRNA at levels lower than that of normal tissue. In contrast, in all three commonly used prostate cancer cell lines (LNCaP, PC-3 and DU 145), the levels of hPRLR mRNA were found to be down-regulated relative to that of normal prostate tissue. Of nine normal human tissues tested, we found that the uterus and the breast have the highest levels of hPRLR mRNA, followed by the kidney, the liver, the prostate and the ovary. The levels of hPRLR mRNA were the lowest among the trachea, the brain and the lung.

The prolactin receptor (PRLR) belongs to the cytokine receptor superfamily. PRLR consists of three domains: the extracellular ligand binding domain, the transmembrane domain and the proline-rich cytoplasmic domain. Following PRL and PRLR interaction, signal transducers and activators of transcription (STATs) are ultimately phosphorylated prior to binding to PRL-responsive promoter elements in the nucleus resulting in PRL action (1,3,5). The evidence linking PRL to breast cancer development has been drawn, in part, from findings of higher PRLR levels in cancerous tissues (6,9,10,12). Experimentally, over-expression of PRL in mice results in a high incidence of mammary tumors. In humans, there is a positive correlation between PRLR, estrogen receptor (ER) and progesterone receptor levels, and it is known that sex steroid hormones and PRL interact synergistically to initiate cancerous growth within mammary tissue (9). There is growing evidence that PRL may also play a role in early transformation events involved in prostate cancer (4), and that PRLR expression is altered in some neoplasms of the prostate (7). More importantly, the PRL antagonist hPRL-G129R, which blocks PRLR signal transduction, appears to induce breast cancer cell apoptosis (2). Therefore, we found it of interest to quantitate PRLR mRNA levels of breast and prostate human cancer cell lines and compare these directly to normal tissue levels. Ultimately, this information will be useful in the selection of cell lines for PRL-related studies based on PRLR status.

*Correspondence: Dr. Wen Y. Chen, 900 W. Faris Road, Oncology Research Institute, Greenville Hospital System, Greenville, SC 29605 Phone: (864-455-1457; Fax (864) 455-1567; Email: wchen@ghs.org

Materials and Methods

Cell lines and tissues: The following human cancer cell lines were obtained from the ATCC and maintained under the conditions recommended. We collected eleven human breast cancer cell lines (MCF-7, T-47D, MDA-MB-134, BT-483, BT-474, MDA-MB-453, MDA-MB-468, BT-549, MDA-MB-436, MDA-MB-157, and MDA-MB-231); three prostate cancer cell lines (LNCaP, PC-3, and DU 145); and the HeLa cell line. Seven tissue total RNA preparations were obtained from Clontech Lab, Inc. (adult brain, kidney, liver, lung, trachea, uterus and prostate), and two from Stratagene, Inc. (adult breast and ovary).

Real-time quantitative PCR: A one-step real time reverse transcription (RT) PCR technique was used to determine relative expression levels of PRLR mRNA using the ABI Perkin Elmer Prism 7700 Sequence Detection System (Applied Biosystems). For analyses from cell cultures, total RNA was isolated from 70-90% confluent cell cultures, using the RNAqueous (Ambion) RNA isolation kit following the recommended protocol. The reaction mix included a 200 nm final concentration of both forward (derived from exon 7: 5′agaccatggatactggagta-3′) and reverse (derived from exon 9: 5′ggaaagatgcaggtcaccat-3′) PRLR-specific primers, and a 100 nm final concentration of the PRLR specific probe (5′tctgctgtcatctgtttgatta-3′) labeled with FAM reporter fluorescent dye; these primers were designed for amplification of all three isoforms of PRLR. A one-step reaction mixture provided in the TaqMan^R Gold RT-PCR Kit (PE Applied Biosystems) was used for all amplifications (5.5 mM MgCl2, 50 mM KCl, 0.01 mM EDTA, 10 mM Tris-HCl pH 8.3, 300 μM deoxyATP, 300 μM deoxyCTP, 300 μM deoxyGTP, 600 μM deoxyUTP, 0.025 U/ml AmpliTaq Gold DNA polymerase, 0.25U/ml MultiScribe Reverse Transcriptase, 0.4 U/ml RNase inhibitor).

Cycle parameters for the one-step RT-PCR included a reverse transcription step at 48°C for thirty minutes, followed by 40 cycles of 95°C denaturation and 60°C annealing/extension. Four hundred to 1500 nanograms of total RNA were used per reaction; the housekeeping gene β-actin was used for internal normalization. For analyses of PRLR in normal tissues, 100 nanograms of commercially-prepared total RNA were used per reaction. Each reaction was carried out in triplicate and repeated at least three times. Data were expressed as the means ± SE.

Results and Discussion

Our results from RT-PCR demonstrate that T-47D cells express the highest levels of PRLR mRNA (Figures 1 and 2) among the cell lines tested. The levels of PRLR mRNA in breast cancer cell lines are much higher than those of prostate cancer cells (Fig. 3). PRLR mRNA was not detectable in HeLa cell RNA preparations (Fig. 3).

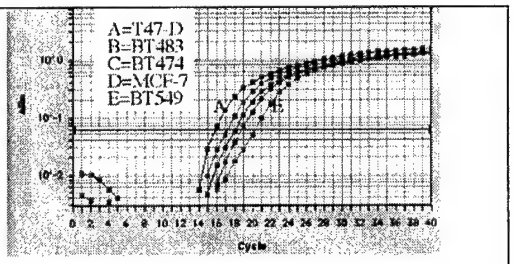

Fig. 1. Real-time RT-PCR analysis for PRLR mRNA in five breast cancer cell lines (A-E, from left to right as indicated).

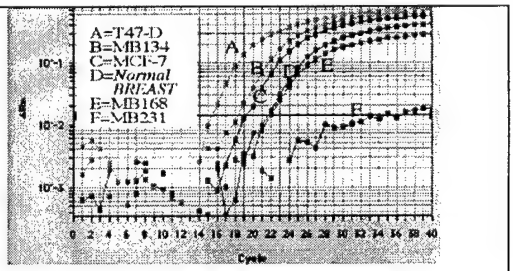

Fig. 2. Real-time RT-PCR analysis for PRLR mRNA in breast cancer cell lines (A-C), normal breast tissue (D) and MDA-MB-468 (E), and MDA-MB-231 (F).

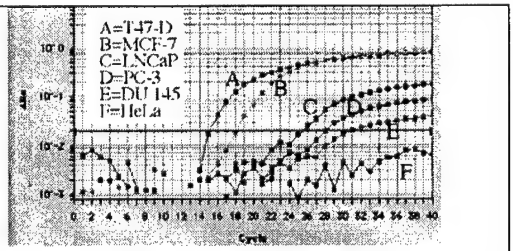

Fig. 3. Real-time RT-PCR analysis for PRLR mRNA in breast cancer cell lines (A, B), prostate cancer cell lines (C-E), and the HeLa cell line (F).

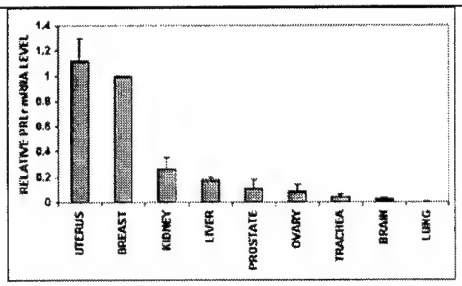

Fig. 4. Comparison of relative PRLR mRNA levels in nine normal human tissues. y-axis, fold difference.

Within the panel of normal tissues, uterus and breast expressed the highest levels of PRLR mRNA (Fig. 4). We set the expression level from breast tissue to 1, to allow internal comparisons between tissues. We found that PRLR mRNA expression from the kidney was surprisingly high, suggesting an important role for PRL in this tissue.

In order to directly compare the expression levels of PRLR between the cell lines and tissue preparations, RT-PCR reactions were carried out using 100 ng of total RNA of normal mammary or prostate tissues and 100 ng of total RNA from several breast or prostate cancer cell lines. We found that the PRLR mRNA expression level of normal breast tissue was comparable to that of the cell line MDA-MB-468 (Fig. 2). Therefore, PRLR mRNA expression levels in MDA-MB-468 cells were used to normalize relative expression level from all cell lines by adjusting all β-actin values to β-actin amplification levels from one ug of MDA-MB-468 total RNA. A graphical representation and summary table of these findings are presented in Figure 5 and Table 1.

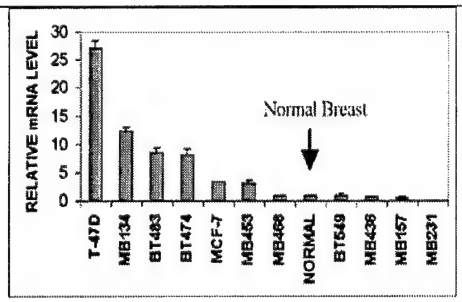

Fig. 5. Comparison of relative PRLR mRNA levels in eleven breast cancer cell lines and normal breast tissue, normalized to 100 ng of total RNA. y-axis, fold difference.

The results using this method were compared to those published earlier in which Northern blotting methods were used to determine relative hPRLR mRNA levels in human breast cancer cell lines (Table 2). Although the two methods generated similar values in most cases, we were able to detect PRLR mRNA expression in cell lines that had previously been noted to lack PRLR expression (MDA-MB-468 and MDA-MB-436). The finding of higher expression levels of PRLR mRNA in four cell lines (T-47D, MDA-MB-134, BT-483 and BT-474) is consistent with the findings of Ormandy

et al. (9). Our findings are also consistent with those of Shiu et al. (11), in which PRLR numbers were directly calculated in a relatively limited panel of breast cancer cell lines.

Table 1. Relative hPRLR mRNA Levels in Human Cancer Cell Lines.

Cell Lines	Fold Difference (± S.E.)
T-47D	27.20 (1.24)
MDA-MB-134	12.45 (0.55)
BT483	8.62 (0.76)
BT474	8.13 (1.03)
MCF-7	3.45 (0.06)
MDA-MB-453	3.17 (0.55)
MDA-MB-468	**1.0***
BT549	**1.0 (0.28)**
MDA-MB-436	0.69 (0.07)*
MDA-MB-157	0.62 (0.07)
LNCaP	0.006 (0.0005)
PC-3	0.002 (0.0002)
MDA-MB-231	0.0017 (0.0064)
DU145	0.00032 (0.00001)
HeLa	not detected

*not detected by Northern blotting methods

It should be noted that although the expression level of PRLR in the normal prostate tissue is moderately high, all three commonly used prostate cancer cell lines expressed extremely low but detectable levels of PRLR mRNA (Fig. 6), ranging from approximately 165 fold lower (LNCaP), and 460 fold lower (PC-3) to 3,100 fold lower (DU 145) than MDA-MB-468 levels (Table 1). We are unsure if down-regulation of PRLR is a common phenomenon of prostate cancer. In any case, one should be aware of lower PRLR levels in these cell lines relative to normal prostate tissue (Fig. 6) when choosing these prostate cancer cell lines as study models.

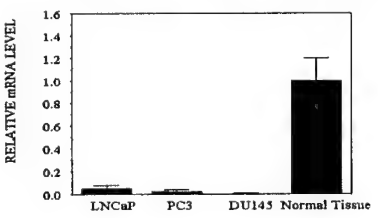

Fig. 6. Comparison of relative PRLR mRNA levels of three prostate cancer cell lines and normal prostate tissue, normalized to 100 ng of total RNA. y-axis, fold difference.

Real-time quantitative PCR is a method proving to be invaluable in the analysis of a number of receptors involved in breast cancer and its metastasis, including prolactin and chemokine receptors (8). Although normal breast tissue expressed the second highest level of PRLR mRNA of the tissue samples, this level was less than a twentieth that of the malignant cancer cell line T-47D, and well below levels of five other mammary cancer cell lines, supporting a growing body of evidence that increased PRLR expression and prolactin activity contribute to mammary carcinoma (3, 9,10,12-15).

Table 2. Relative hPRLR mRNA Levels: Comparison Between Two Studies

Cell Lines	Current Study	Ormandy et al. (9)
T-47D	7.9	4.0
MDA-MB-134	3.6	5.2
BT483	2.5	4.0
BT474	2.4	2.3
MCF-7	**1.0**	**1.0**
MDA-MB-453	0.9	0.7
MDA-MB-468	0.3	ND*
BT549	0.3	0.6
MDA-MB-436	0.2	ND*
MDA-MB-157	0.2	0.4
MDA-MB-231	0.0005	not done

*not detected by Northern blotting methods

Acknowledgments

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Appendix D.

Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling.

Beck MT, Holle H, Chen WY

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Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling

Michael T. Beck[1], Lori Holle[2], and Wen Y. Chen[1, 2]

[1]Department of Microbiology and Molecular Medicine Clemson University, Clemson, SC, USA

[2]Oncology Research Institute Greenville Hospital System, Greenville, SC, USA

Address correspondence to: Dr. Wen Y. Chen, 900 West Faris Road, Greenville, SC 29605, USA. E-mail: wchen@ghs.org

Key Words: subtraction hybridization, digoxigenin (DIG), and cDNA microarray

ABSTRACT

PCR subtraction hybridization has been used effectively to enrich and single out differentially expressed genes. Identification of these genes, however, by means of cloning and sequencing individual cDNAs is a tedious and lengthy process. In this report, an attempt has been made to combine the use of PCR select cDNA subtraction hybridization and cDNA microarrays to identify differentially expressed genes using a non-radioactive chemiluminescent detection method. mRNA from human prolactin (hPRL) or human prolactin antagonist (hPRL-G129R) treated and non-treated breast cancer cells was isolated and cDNAs were synthesized and used for the PCR subtraction to enrich the differentially expressed genes in the treated cells. The PCR amplified and subtracted cDNA pools were purified and labeled using the digoxigenin (DIG) method. Labeled cDNAs were hybridized to a human apoptosis cDNA microarray membrane and identified by chemiluminescence. The results suggest that the strategy of combining all three methods will allow for a more efficient, non-radioactive way of identifying differentially expressed genes in target cells.

INTRODUCTION

Our previous studies have shown that hPRL demonstrates a stimulatory effect on human breast cancer cell proliferation (1). We have also reported that an hPRL mutant with a single amino acid substitution mutation at position 129 (hPRL-G129R) acts as an hPRL receptor antagonist on human breast cancer cells (1). Further investigation of hPRL-G129R demonstrated that its inhibitory effects on breast cancer cells are through the induction of apoptosis (1). However, the exact mechanism of hPRL-G129R induced apoptosis is still awaiting further investigation.

To study the physiological mechanisms of different cell types and of cells under different conditions PCR subtraction hybridization has been used widely over the years (4-6). This technique gives a representation of differentially expressed genes from one group of cells as compared to another. The theory behind the technique is very simple. It first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the "tester" and the reference cDNA is referred to as the "driver". Both "tester" and "driver" cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The "tester" cDNAs are then hybridized with "driver" cDNAs, which have no adaptors on their ends. Suppression PCR, using the adaptors as primers, is then performed to allow exponential amplification of the differentially expressed genes.

To identify these genes by use of conventional methods such as cloning, sequencing, and northern blot analysis is a tedious and expensive process (6, 9). Recently, the vast emergence of the cDNA microarray techniques greatly expands the ability of researchers to identify previously cloned sequences in pools of cDNAs. This technique has proved to be an essential tool when trying to identify what genes are responding to a certain condition (6, 9). One of the drawbacks of using directly isolated

mRNA/cDNA as probes in screening commercial membranes is a high noise-to-signal ratio (2). In this study, we combine the use of suppression subtractive hybridization to enrich differentially expressed cDNAs, upon treatment with hPRL and hPRL-G129R. We then will identify the differentially expressed cDNAs by use of microarray technology. Also, nucleotide hybridization detection without the use of radioisotopes is of interest to many researchers interests (3, 7, 11). Therefore we employed the use of a non-radioactive chemiluminescent system, digoxigenin (DIG), to label the cDNAs as an alternative way of identifying genes on the microarray membrane.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

The T-47D human breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). T-47D cells were maintained in phenol red-free RPMI 1640 media (Life Technologies Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Inc., Rockville, MD, USA) and grown at 37°C in an atmosphere containing 5% CO_2. Prior to treatment with hPRL or hPRL-G129R, cells were split into three groups (10 T75 flasks were used for each group) and depleted with RPMI 1640 supplemented with 10% Charcoal/Dextran treated FBS (CSS) for 6 days until cells reached 80% confluency. After depletion, approximately 10^8 cells from each group were treated with either 500 ng/ml of hPRL (Tester A) (hPRL was kindly supplied by Dr. A.F. Parlow, National Hormone and Pituitary Program, NIH, USA) in RPMI 1640 supplemented with 1% CSS or 500 ng/ml of hPRL-G129R (Tester B) (hPRL-G129R was produced in our laboratory, 8) or cultured with 1% CSS alone as the untreated control (Driver). Cells were treated for 48-h and then mRNA was isolated in the following section.

PCR cDNA Subtraction Hybridization

mRNA isolation was performed using the Micro-Fast Track 2.0 kit from Invitrogen Corp. (Carlsbad, CA, USA) according to manufacture's instructions. RNA yield was determined by measuring absorbance at 260nm. Subtractive hybridization was performed using the PCR Select™ cDNA Subtraction Kit from Clontech Laboratories, Inc. (Palo Alto, CA, USA). Briefly, 10 µg of mRNA was used for synthesizing Tester A, Tester B and Driver cDNAs. Restriction enzyme digests of the cDNAs, adaptor ligation of the testers, and two rounds of hybridization between "tester" and "driver" were carried out following manufacture's instructions. After hybridizations were complete

primary PRC was used to amplify products. Conformation of the subtractions was performed on the primary PCR products using the primers for the housekeeping gene, G3PDH, supplied in the kit.

Generation of enriched cDNAs, which will be labeled and used as probes for screening the microarray, were prepared with a secondary PCR reaction. Four 50 µl PCR reactions were performed and pooled. cDNAs were purified using QIAquick PCR Purification Kit from QIAGEN Inc. (Valencia, CA, USA). DNA yield was determined by measuring absorbance at 260 nm.

Labeling of cDNA Probes

Purified cDNAs from the PCR subtraction hybridization were random primed labeled with digoxigenin-dUTP, alkali labile (DIG) DNA Labeling Kit (Roche Molecular Biochemical's, Mannheim, Germany). Three µg of cDNA was labeled according to the manufacture's protocol and incubated for >20 h. An overnight incubation was performed, as previous results have demonstrated, to ensure an efficient yield of newly synthesized DIG-labeled DNA.

cDNA Microarray

Atlas™ Human Apoptosis Arrays from Clontech Laboratories, Inc. (Palo Alto, CA, USA) containing all currently known apoptosis related genes on a nylon membrane were prehybridized with DIG Easy Hyb solution (Roche Molecular Biochemical's, Mannheim, Germany) at 37°C for 2 h in a hybridization incubator with gentle rotation. Three µg of DIG-labeled probes were purified and resuspended in 20 µl of dH_20. The probes were boiled for 10 min and quickly chilled on ice for ≥5 min. After prehybridization 20 µl of DIG-labeled probe was added to the microarray membrane in which 5 ml of fresh DIG Easy Hyb had been added. Membranes were hybridized overnight at 68°C in a hybridization incubator with gentle rotation. The following day, membranes were washed at 38°C for

two times (5 min/wash) in 2X SSC, 1% SDS, and at 68°C for two times (15 min/wash) in 0.1X SSC, 0.5% SDS.

Chemiluminescent Detection

Hybridized Atlas™ membranes were developed using the DIG Luminescent Detection Kit (Roche Molecular Biochemical's, Mannheim, Germany) according to the manufacture's specifications. CSPD® was used as the chemiluminescent substrate. After 5 min of incubation with CSPD® membranes were wrapped in plastic wrap, placed in an autoradiography cassette, and incubated at 37°C for 15 min. The membrane was then exposed to Kodak Biomax™-MR film at room temperature for various amounts of time to obtain an optimal exposure.

RESULTS AND DISCUSSION

In this report, we successfully combined three proven effective commercial available methods to profile genes in breast cancer cells in response to various treatments using non-radioactive techniques.

When performing the mRNA isolation we have found that it is important to start with at least 10^8 cells in order to obtain sufficient mRNA for completing the experiment. We have found that a minimum of 10 μg of mRNA should be used (instead of 2 μg as recommended) to produce the optimal amount of cDNAs for the remainder of the experiment. Once the primary and secondary PCR's are completed, an efficiency test must be performed to verify the subtraction efficiency. As shown in Figure 1, after two separate PCR runs were performed, the G3PDH was greatly reduced in the subtracted samples as compared to the unsubtracted.

In order to obtain enough cDNAs to be used for labeling and probing of the microarray, we recommend pooling multiple secondary PCR reactions together when making the cDNA probes. Figure 2 shows the results of the cDNA enrichment reactions. The cDNA shown on the gel represents one tenth of the purified pool of cDNAs obtained from the final PCR product (keep in mind that housekeeping genes such as G3PDH could not be amplified after subtraction, see Figure 1). A total of 3 μg of amplified final PCR produced cDNAs was used for labeling with DIG to increase the amount of genes to be properly labeled.

To search for any apoptosis related genes present in the cDNA pools after hPRL or hPRL-G129R treatments, an Atlas™ human apoptosis cDNA microarray containing 205 apoptosis related genes was used. Figure 3 shows the results after hybridization with the DIG labeled cDNAs. There are distinct differences found with the treatment of either hPRL or hPRL-G129R. Many apoptosis related genes are being expressed in the hPRL-G129R treated cells, as would be expected since previous

experiments showed it to induce apoptosis. Various caspases are shown in lane 11. There is no presence of these genes in the hPRL treated cDNAs as shown in Figure 3.

These results prove that by combining the use of PCR Select cDNA subtraction hybridization, cDNA microarrays, and chemiluminescent detection one is able to detect and identify differentially expressed genes. This technique is valuable in that it can be performed with any desired cell lines, with any treatment of the researcher's choice, and probed using any variation of cDNA microarray membranes. These methods together will allow researchers the ability to target and study specific differentially expressed genes at a cost efficient as well as in an environmental friendly manner.

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Figure Legends

Figure 1. Efficiency test of the PCR select subtraction hybridization. The housekeeping gene G3PDH (~400 bp fragment) amplified for 12 cycles with primers provided from PCR-Select cDNA Subtraction Hybridization kit on a 1% agarose/EtBr gel. Lane 1 is Lambda DNA/Hind III digested molecular weight marker. Lane 2 and 4 are subtracted Testers A and B respectively, and lanes 3 and 5 are the unsubtracted tester controls.

Figure 2. Analysis of enriched cDNAs from PCR select subtraction hybridization. After the secondary PCR a sample of the purified hPRL specific cDNAs (Lane 2) and hPRL-G129R specific cDNAs (Lane 3) was run on a 1% agarose/EtBr gel. Lane 1 is Lambda DNA/Hind III digested molecular weight marker.

Figure 3. Microarray hybridized with enriched subtracted cDNAs. A human apoptosis cDNA microarray was hybridized with DIG labeled, hPRL specific cDNAs (A) and hPRL-G129R specific cDNAs (B). A section of the hPRL specific array (a) and hPRL-G129R specific array (b) was enlarged for comparison purposes. We have identified that cDNAs represented by 10F in the hPRL treated cancer cells and various others in the hPRL-G129R treated cells are of special interest.

Appendix E.

Six abstracts in recent meeting presentations

In Vivo Studies of the Anti-tumor Effects of a Human Prolactin Antagonist, hPRL-G129R in Nude Mice

Chen, N.Y., Li, W., Cataldo, L., Sticca, R.P., Wagner, T.E. and Chen, W.Y.
Oncology Research Institute, Greenville Hospital System, Greenville, SC 29605, and Department of Microbiology and Molecular Medicine, Clemson University, Clemson, SC 29634

Human breast cancer is the predominant malignancy and leading cause of cancer death in women in Western society. In our previous studies, we demonstrated that a mutated human prolactin (hPRL) with a single amino acid substitution at position 129 (hPRL-G129R) was able to inhibit human breast cancer cell proliferation via the induction of apoptosis. We also showed that the inhibitory effect of hPRL-G129R on human breast cancer cells is probably through the inhibition of STAT3 phosphorylation, induction of Caspase 3 and/or modulation of TGFs. In this study, we report the results of using hPRL-G129R as a therapeutic agent in nude mice bearing T-47D human breast cancer xenografts. Human PRL and hPRL-G129R used in this study were produced using an E. coli (pET22b) protein expression system (Novagen, Madison WI). Protein products in the form of inclusion bodies were harvested from E coli lysates and resuspended in 8M urea for refolding. After refolding, the samples were purified using a FPLC/Q-Sepharose anionic exchange column. The purity of the protein products is over 90%, based on SDS-PAGE and silver staining results. Since the *in vivo* half-life of hPRL is less than two hours, proteins were formulated with Matrigel (Becton Dickinson, Bedford, MA) before injection as an alternative way of slow releasing hPRL and hPRL-G129R *in vivo*. The half-life of hPRL or hPRL-G129R after formulated with Matrigel was found to be longer than 8 hrs. Thirty BalbC/nude mice were injected with $5x10^6$ T-47D cells and implanted s.c. with slow releasing E2 pellets (1.7 mg/60 day, Innovative Research of America, Inc. Sarasota, Fl). One week after tumor cell inoculation, the mice were randomized into three groups, and injected daily (5 times/week) with 100ul of Matrigel (control), hPRL/Matrigel (100ug/100ul), or hPRL-G129R/Matrigel (100ug/100ul) for 7 consecutive weeks. Tumor growth was monitored weekly. At the end of the 7 week period of treatment, we found that hPRL simulated the *in vivo* growth of T-47D cells (mean tumor volume was 202 +/- 62 SEM mm^3 as compared to 124 +/- 31 SEM mm^3 in control mice). More importantly, daily injection of hPRL-G129R inhibited the tumor growth (mean tumor volume was 79 +/- 32 SEM mm^3). We are currently investigating more effective methods for the delivery of hPRL-G129R. We hope that the hPRL antagonist can be used to improve the outcome of human breast cancer treatment in the near future.

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(AACR 92nd Annual Meeting March 24-28, 2001)

ENHANCEMENT OF THE INHIBITORY EFFECTS OF SUPPRESSOR OF CYTOKINE SIGNALING 3 (SOCS3) PROTEIN IN CANCER CELLS BY VP22

Q. Yuan[2], S.K. Peirce[2], R.B. Westberry[2] and W.Y. Chen[1,2]. [1]Oncology Research Institute, Greenville Hospital System, Greenville, SC, United States, [2]Microbiology and Molecular Medicine, Clemson University, Clemson, SC, United States

SOCS3 protein is a novel regulator of intracellular signaling. There is growing evidence to suggest that SOCS3 inhibits STAT3 (Signal Transducer and Activator of Transcription 3) which is reported to be constitutively activated in many cancers and transformed cell lines. The herpes simplex virus protein VP22 has the unusual properties of both cell-contact independent intercellular transport and nuclear targeting. In this study, the inhibitory effects of SOCS3 and the nuclear targeting function of VP22 were combined to create a fusion protein (VPSOCS3). A murine cell line, B16 melanoma, and the human T-47D breast cancer line were used in this study. After PCR and cloning of the SOCS3 cDNA, it was inserted into CMV or CMV-VP22 expression vectors (Invitrogen). B16 and T-47D cells were transfected with these constructs and stable cell lines were established. The growth rates of cell lines expressing vector only, SOCS3, or VPSOCS3 were compared using a cell proliferation assay, and growth inhibition by SOCS3 in B16 cells was shown to be approximately 24%. VPSOCS3 expression enhanced the inhibitory effect of SOCS3 in B16 cells to 49%, an inhibition double that of SOCS3 alone. Similar inhibitory patterns were observed when SOCS3 and VPSOCS3 were expressed in T-47D cells; SOCS3 expression resulted in a 25% inhibition, and VPSOCS3 resulted in a 57% inhibition. These findings suggest that targeting of SOCS3 to the nucleus by VP22 enhances the inhibitory effects of SOCS3. We further investigated the intercellular transport ability of VPSOCS3 in B16 and T-47D cells using a mixed-culture proliferation assay. The intercellular trafficking function of the novel fusion protein VPSOCS3 supports its potential as a cancer therapeutic.

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(Endo 2001, Denver June 20-23)

REAL TIME RT PCR ANALYSIS OF RELATIVE PROLACTIN RECEPTOR (PRLR) LEVELS IN HUMAN CANCER CELL LINES.

S.K. Peirce[2], R.B. Westberry[2] and W.Y. Chen[1,2]. [1]Oncology Research Institute, Greenville Hospital System, Greenville, SC, United States, [2]Microbiology and Molecular Medicine, Clemson University, Clemson, SC, United States

The prolactin receptor (PRLR) belongs to the cytokine receptor superfamily. The evidence linking PRL to breast cancer development has been drawn in part from findings of high PRLR levels in cancerous tissues. Experimentally, activation of the PRLR induces mammary tumors in mice. In humans, there is a positive correlation between PRLR, estrogen receptor (ER) and progesterone receptor levels, and it is known that sex steroid hormones and PRL interact synergistically to initiate cancerous growth within mammary tissue. There is growing evidence that PRL may also play a role in early transformation events involved in prostate cancer, and that PRLR expression is altered in some neoplasms of the prostate. It is therefore of interest to compare the PRLR status of breast and prostate cancer cell lines, as well as other human cancer cell lines. In this study, we used a one-step real time reverse transcription PCR technique to determine relative expression levels of PRLR mRNA in ten human breast cancer cell lines, three prostate cancer and several other cell lines; the housekeeping gene b-actin was used for internal normalization. The results using this method were compared to those published earlier in which Northern blotting methods were used to determine relative hPRLR mRNA levels. Of the human breast cancer cell lines examined, T47D was found to have the highest level of PRLR: 7.89 (+/- 0.36 SE) fold higher than that of MCF-7 cells. MB157 cells expressed the lowest levels, 0.18 (+/- 0.02 SE) relative to MCF-7 cells. Expression levels in the prostate cell lines were very low but detectable, ranging from approximately 700-fold lower (LNCaP) to 26,000-fold lower (DU145) than MCF-7 levels; HeLa PRLR expression was not detectable. Ultimately, this information will be useful in the selection of model cell lines based on PRLR status.

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PROFILING OF APOPTOSIS RELATED GENES RESPONDING TO PROLACTIN AND ITS ANTAGONIST IN HUMAN BREAST CANCER CELLS.

M.T. Beck[1], L. Holle[1] and W.Y. Chen[1,2]. [1]Microbiology and Molecular Medicine, Clemson University, Clemson, SC, United States, [2]Oncology Research Institute, Greenville Hospital System, Greenville, SC, United States

It has been shown that human prolactin (hPRL) serves as a survival/growth factor for human breast cancer cells. In our previous studies we have demonstrated that a hPRL antagonist, hPRL-G129R, was able to inhibit breast cancer cell proliferation via the induction of apoptosis. We have also suggested that possible mechanisms of hPRL-G129R induced breast cancer cell apoptosis were through modulation of TGFs, caspases, or STATs. This study focuses on the expression profile of genes related to apoptosis in human breast cancer cells upon treatment with hPRL or hPRL-G129R. We first utilized the technique of PCR-Select cDNA subtraction hybridization (Clontech, Inc.) to enrich differentially expressed genes from T47D human breast cancer cells treated with either hPRL or hPRL-G129R. The enriched and differentially expressed cDNA pools (cDNAs from treated cells subtracted by that of untreated cells) were labeled with digoxigenin (DIG) and hybridized to a nylon membrane that contains most known apoptosis related genes (Atlas Human Apoptosis Microarray from Clontech, Inc.). Our preliminary results from hPRL treated T47D cells revealed that out of the 205 apoptosis related genes only one gene, bcl-2, was up regulated in response to hPRL (bcl-2 is known as an apoptosis suppressor). Many apoptosis related genes, in particular various caspases (3 and 7), Fas-activated serine/threonine (FAST) kinase, members of the Tumor Necrosis Factor (TNF) family, and E2F were up regulated in hPRL-G129R treated T47D cells. This information provides a comprehensive report of genes responsible for hPRL-G129R induced apoptosis in human breast cancer cells. The results from this study also suggest that hPRL might act as a survival factor by inducing genes involved in suppression of apoptosis. In conclusion, these findings provide a better understanding of the relationship between hPRL/hPRL-G129R and human breast cancer.

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CHARACTERIZATION OF A HUMAN PROLACTIN ANTAGONIST/ GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR FUSION PROTEIN.

L. Holle[2], W. Li[2], N.Y. Chen[1] and W.Y. Chen[1,2]. [1]Oncology Research Institute, Greenville Hospital System, Greenville, SC, United States, [2]Microbiology and Molecular Medicine, Clemson University, Clemson, SC, United States

Granulocyte macrophage colony stimulating factor (GM-CSF) displays potent effects on the immune system, and as a result, has been used in clinical trials for patients with diseases such as AIDS and aplastic anemia. GM-CSF has also been shown by numerous groups to be effective in the treatment of tumors in mice. In order for GM-CSF to be effective in the treatment of a tumor, large quantities of GM-CSF must be given. High serum levels of GM-CSF, however, have serious and even toxic side effects. Previously in our laboratory we have shown that a human prolactin antagonist (hPRLA), hPRL-G129R, was able to inhibit human breast cancer cell proliferation via the induction of apoptosis. Our preliminary results also show that hPRL-G129R is effective in decreasing tumor size in nude mice. In this study we target GM-CSF to breast cancer cells by designing a hPRLA/GM-CSF fusion protein (hPRLA-GMCSF). hPRLA-GMCSF was produced in the form of an inclusion body using the pET22b expression vector and purified using a Q-Sepharose anion exchange column. Silver staining of the purified protein revealed production of a protein with a purity of greater than 90%. Western analyses using antibodies against either hPRL or hGM-CSF confirmed the identity of the fusion protein. A STAT5 tyrosine phosphorylation assay was used to test the PRL receptor antagonistic effects of the fusion protein. The results demonstrated that hPRLA-GMCSF was able to inhibit STAT5 phosphorylation to the same extent as hPRL-G129R. Cell proliferation assays using TF-1 cells, a hGM-CSF dependent cell line, were used to determine the function of the hGM-CSF portion of the fusion protein. The fusion protein was able to stimulate cell proliferation of TF-1 cells with the same potency as that of hGM-CSF alone. In conclusion we were able to produce a bifunctional fusion protein which we hope will be useful in cancer therapy.

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A NOVEL DESIGN OF TARGETED ENDOCRINE AND CYTOKINE THERAPY FOR HUMAN BREAST CANCER

G.R. Zhang[2], W. Li[2], L. Holle[2], N.Y. Chen[1] and W.Y. Chen[1,2]. [1]Oncology Research Institute, Greenville Hospital System, Greenville, SC, United States, [2]Microbiology and Molecular Medicine, Clemson University, Clemson, SC, United States

The aim of this study is to combine the essence of endocrine therapy (human prolactin antagonist, hPRLA) and immune therapy (interleukin 2, IL-2) in the design of a targeted endocrine/cytokine fusion protein, hPRLA-IL2, to treat human breast cancer. This novel approach utilizes the specific interaction between hPRLA and hPRL receptors (PRLR), thus targeting the fusion protein to the malignant breast tissues that have been shown to contain high levels of PRLR. The localized bi-functional fusion protein then blocks the signal transduction induced by hPRL as well as activates T lymphocytes at the tumor site. The targeted IL-2 approach should greatly reduce the systemic concentration of IL-2. The fusion protein was produced using a bacterial expression system (Novagen Inc.), FPLC purified using a Q sephorose XL column (Pharmacia Inc) and confirmed by immnoblotting analysis. The IL-2-like activity of the fusion protein was tested by a HT-2 cell proliferation assay. The antagonistic activity of the fusion protein was tested using a STAT phosphorylation assay. Our results demonstrated that the fusion protein is as effective in the inhibition of hPRL-induced STAT phosphorylation as hPRLA. The potency of the fusion protein on HT-2 proliferation is similar to that of IL-2. Our preliminary in vivo results demonstrated that the fusion protein has a relatively long half-life as compared to hPRL or IL-2. We further tested the in vivo anti-tumor activities using a syngenic Balb/c mouse tumor model following s.c. injection of EMT6 mouse breast cancer cells. After daily injection of either fusion protein (50ug/mouse, i.p.) or saline for two weeks, the fusion protein maintained a steady level in serum (~50ng/ml). The tumor growth in the treated group was significantly reduced relative to that of the control group. At the end of the two weeks treatment, the tumor weight in the treated group was only 50% as compared to that of the control group. We hope that this novel fusion protein will contribute significantly to human breast cancer therapy.

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